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ADAPTATION OF THE METHOD OF LOWRY AND LOPEZ TO THE ESTIMATION OF INORGANIC AND ORGANIC PHOSPHATE IN PLANT EXTRACTS[1]

BY E. R. WAYGOOD[2]

Abstract

The method of Lowry and Lopez using ascorbic acid as the reducing agent with the Fiske–Subbarow reagents at pH 4.0 is proposed for the estimation of phosphate in plant extracts. The method has been reinvestigated with respect to its adaptation to the Coleman Universal spectrophotometer, Model 14, to suitability of wave length, pH range, reagent concentration, rate of reaction, color stability, and the effect of silicate. The experiments, in general, confirm those of Lowry and Lopez. A wave length of 650 mμ has been employed. No blue color is formed in the reagent blank between a pH of 3.1 and 4.2; accordingly a pH of 4.0, maintained by a sodium acetate – acetic acid buffer, has been used. A concentration of 0.25% ammonium molybdate and 0.1% ascorbic acid has been shown to be optimum for maximum color development in concentrations of phosphate up to about 25 μgm. P in the final volume. The reduction of phosphomolybdic acid is complete in about five minutes, and the method is characterized by a high degree of color stability. Silicate interferes with the reaction. Where the plant has a liberal silicate economy the cell wall material, with which the silicon is associated, must be removed from the extract. The method has been applied to the estimation of phosphate liberated from organic combination by hydrolysis for specified times in 1*N* hydrochloric acid. Total phosphate is estimated by wet ashing with 60% perchloric acid. Recovery tests of known amounts of inorganic phosphate and glucose-1-phosphate added to wheat leaf and seedling extracts give an absolute error within 2%, which is considered permissible. A balance sheet of phosphate fractions in the supernatant, residue, and whole juice of wheat leaves is discussed.

Introduction

Methods for the determination of phosphate in plant and animal extracts are based on the formation of a blue coloration (molybdenum blue) by the action of a reducing agent on a phosphomolybdic acid complex. The method of Fiske and Subbarow (4) in which 1-amino-2-naphthol-4-sulphonic acid is used as the reductant has been widely adopted. Such modifications as have been applied to the method, in general, refer to the use of other and more suitable reducing agents (1, 5, 7, 8, 10). These investigators have employed stannous chloride (8), methyl-*p*-aminophenol sulphate (elon) (5, 7), 2,4-diaminophenol hydrochloride (amidol) (1), and ascorbic acid (10) as reducing agents in efforts to improve the color stability and acid tolerance of the

[1] *Manuscript received June 4, 1948.*
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method. Stannous chloride appears to be the most sensitive, but it has a very narrow range of acid tolerance and is quite unstable in dilute solution (5, 8). The sulphonic acid has a slightly wider range of acid tolerance (pH 0.5 to 0.6), but color development occurs apparently indefinitely (5, 7). Elon has proved to be more satisfactory, the sensitivity of the method is decreased, but its acid tolerance (pH 0.2 to 0.8) is even greater than with the sulphonic acid (7). Its solution is stable, and color stability is claimed after 30 to 60 min. (5, 7). Lowry and Lopez (10) used ascorbic acid as the reducing agent and showed that the acid safety limits for the reduction of phosphomolybdic acid were between a pH range of 0.4 to 0.9 and pH 2.8 to 4.6. They used the latter range in their estimations and showed that the higher pH conferred a greater degree of stability upon labile phosphate esters. Arney (2), after a preliminary separation of inorganic phosphate used the Fiske and Subbarow method for the estimation of phosphate cleaved from labile and resistant esters in barley extracts. Berenblum and Chain (3) modified the method using isobutyl alcohol as the reaction phase, in which they claimed the reduction of phosphomolybdic acid is more rapid and complete. Allen (1) found that their method was not applicable to extracts from certain higher plants and further modified the method for general use. Pons and Guthrie (11) have also investigated the validity of this method and adapted it for use with a spectrophotometer for the determination of plant materials high in protein.

Preliminary experiments were undertaken in this laboratory in order to determine which of these methods was best adapted to a study of phosphorylations and dephosphorylations in extracts and isolated enzyme systems of plants.

In the development of a colorimetric method, perhaps the main requirement is stability of color after the reaction has been completed. Since it is impossible to purify biological systems completely, conditions for the reaction to take place must be flexible and must allow for the uncontrollable variation in the natural buffers and interfering substances. Thus complicated methods and exacting procedures, with narrow ranges of acid tolerance, subject to color instability must be avoided if possible.

Initial tests conducted on the Fiske and Subbarow reagents using stannous chloride and 1-amino-2-naphthol-4-sulphonic acid confirmed the narrow range of acid tolerance at the low pH and the instability of the color developed. Elon was more satisfactory on our plant extracts, but the present author was unable to confirm the color stability claimed to be attained after 30 min. by Gomori (5) and after one hour by Holmes and Motzok (7) using this reagent.

The use of ascorbic acid as reducing agent was investigated and by virtue of its use it was found that the method conformed more rigidly to the requirements previously defined for a colorimetric method. The procedure of Berenblum and Chain (3) was tested, but the author was unable to obtain satisfactory results and hence it was decided to make a more detailed investigation

into the method of Lowry and Lopez (10) with a view to determine its applicability to plant extracts. Many determinations have since been made on extracts from parts of wheat, tomato, and onion plants using the adapted method and these have sufficed to confirm the validity of the method to be discussed.

Experimental Results

The Method on Pure Solutions of Phosphate

Solutions Required

1. Sodium acetate – acetic acid buffer: (0.025N and 0.1N), pH 4.0.
2. Ammonium molybdate: 5% aqueous solution, stored in an opaque, paraffin-wax lined glass stoppered bottle.
3. Ascorbic acid: 1% in acetate buffer (solution No. 1), stored under toluene in an opaque glass stoppered bottle at 4° C.
4. Potassium dihydrogen phosphate: stock solution, containing 1 mgm. P per ml. (2.195 gm. per 500 ml. water).
5. Solution No. 4 diluted 100 times, contains 10 μgm. P per ml.

The ammonium molybdate solution was found to be quite satisfactory without acidification if stored under the prescribed conditions. The ascorbic acid solution is quite stable for at least four months, if not longer, providing a layer of toluene covers the solution and it is stored in a refrigerator.

The Colorimeter

Any suitable photoelectric colorimeter may be employed. In this study the method has been adapted to the Coleman Universal spectrophotometer, Model 14, using 14–303 cuvettes, matched to ½% transmission. However the method has been used successfully with the Klett-Summerson photoelectric colorimeter with a No. 69 (690mμ) filter in position.

Procedure

Transfer 1 ml. of solution No. 5 (10μgm. P per ml.) to a 10 ml. volumetric flask. Add 2 ml. of acetate buffer (pH 4.0, solution No. 1) and 0.5 ml. of ammonium molybdate (solution No. 2). Shake well. Add 1 ml. of ascorbic acid (solution No. 3), make to volume with distilled water, and after vigorous shaking, allow to stand for at least five minutes. At the same time a blank should be made using distilled water. After five or more minutes transfer the contents of each flask to the matched cuvettes and place in the spectrophotometer set at a wave length of 650 mμ. Adjust the galvanometer so that the blank reads 100% transmittance or zero optical density ($D = -\log T$) and record the percentage transmittance when the phosphate solution is moved into the beam of light. At this wave length setting, the concentration of phosphorus, as will be seen later, is inversely proportional to the logarithm of the percentage transmittance (i.e. $C = -k \log T$) and this reading will constitute a calibration factor for the instrument from zero concentration (100% T) up to and including a concentration of 10 μgm. P measured.

An accurate calibration curve may be prepared by using various concentrations of phosphorus and plotting concentration against the logarithm of the percentage transmittance in the manner shown in Fig. 1.

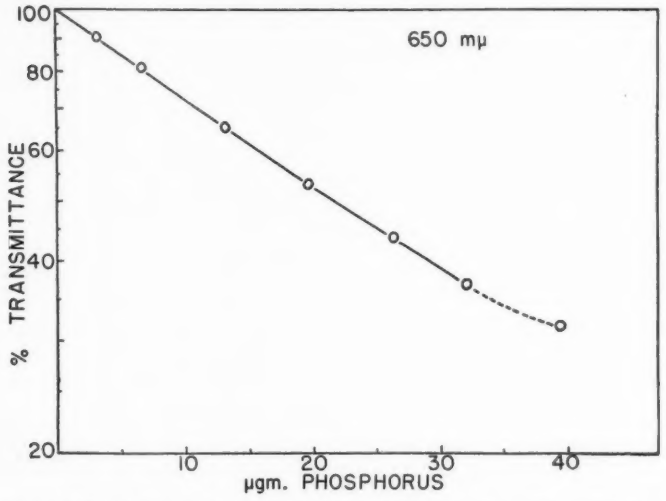

FIG. 1. *Calibration curve of phosphorus using the Coleman Universal Spectrophotometer, Model 14, and showing close conformity to Beer's law ($C = -k \log_{10} T$) at 650 mμ and pH 4.0. Dotted line, nonconformity to Beer's law at this concentration of phosphorus.*

Absorption Spectrum of the Reduced Phosphomolybdic Acid Complex

In order to determine that the wave length of light should be so chosen that the relation between concentration and transmittance conforms to Beer's law ($C = -k \log T$; Fig. 1) the absorption spectrum of the phosphomolybdic acid complex was followed over a range from 325 mμ to 825 mμ. Various concentrations of phosphorus were compared to the reagent blank set at 100% transmittance. The data are graphically represented in Fig. 2. The absorption spectrum of this blank compared to distilled water at 100% transmittance is also shown. The blank itself shows well defined absorption bands in the regions 325 mμ to 450 mμ and past 700 mμ, therefore these parts of the spectrum must be avoided for the colorimetric determination, since it is advisable to reduce to a minimum the absorption due to the reagents and their impurities. Analysis of the data show that Beer's law holds over the range from 500 mμ to 675 mμ, hence it was decided to use the wave length 650 mμ, which tends towards increasing the sensitivity of the method. The calibration curve at this wave length is shown in Fig. 1. It will be observed that there are three parts to this curve. The first up to 20 μgm. P, the second from 20 μgm. P to about 30 μgm. P, and the third above 30 μgm. P.

Deviation from Beer's law occurs at relatively high concentrations i.e. 20 μgm. P to 30 μgm. P, but the deviation is not sufficient to invalidate the

determinations in this region. Beyond a concentration of about 30 μgm. P the deviation is much greater and determination in this region could not be relied upon.

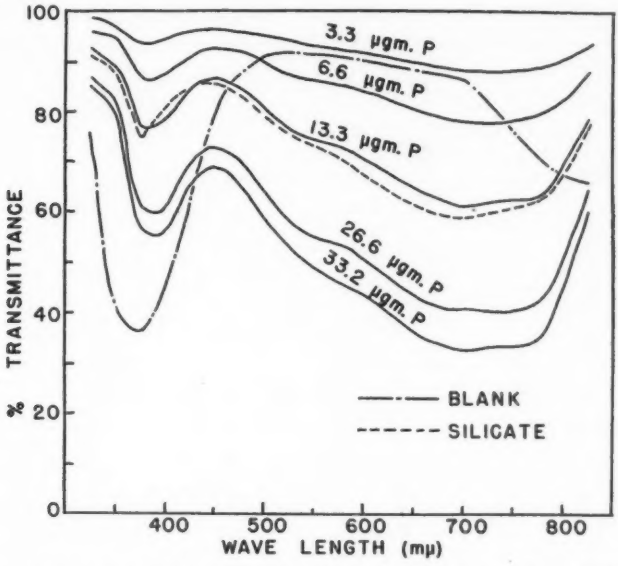

FIG. 2. *Absorption spectra of reduced phosphomolybdic acid at pH 4.0 and various levels of phosphorus. Absorption spectra of the reagent blank and a molybdisilicic acid complex (120 μgm. Si) at pH 4.0.*

Effect of Acidity on the Reduction of Molybdic Acid

Molybdic acid as well as phosphomolybdic is reduced to molybdenum blue at certain pH values. The reduction of molybdic acid is determined by following the color development in the reagent blank at various pH levels. That region of the pH scale wherein no color develops in the blank compared to the blank at pH 4.0 is suitable for the reduction of phosphomolybdic acid. Accordingly the pH of the reagent blank was adjusted to a series of values between pH 0.5 and pH 4.5 by the addition of 10*N* sulphuric acid or 1*N* sodium acetate to the buffer solution No. 1. After readings had been taken of the absorption of the solutions, measured by optical density, the pH of each solution was measured accurately. A graph indicating the relation between pH and the reduction of molybdic acid compared to zero reduction at pH 4.0 is shown in Fig. 3. Reduction of molybdic acid occurs in the range from pH 1.0 to pH 3.1, and since the color was developing continually except in the blank, a specified time of 15 min. was chosen to record the transmittance of the solution. In the pH ranges where no absorption occurred when referred to the reagent blank at pH 4.0, readings were taken after five minutes, by

which time the optical density was stable. If various concentrations of phosphorus were admitted, the color development was proportionately higher in the pH range where no blue color developed in the blank, and considerably higher in the range pH 1.0 to pH 3.1, the latter indicating the combined

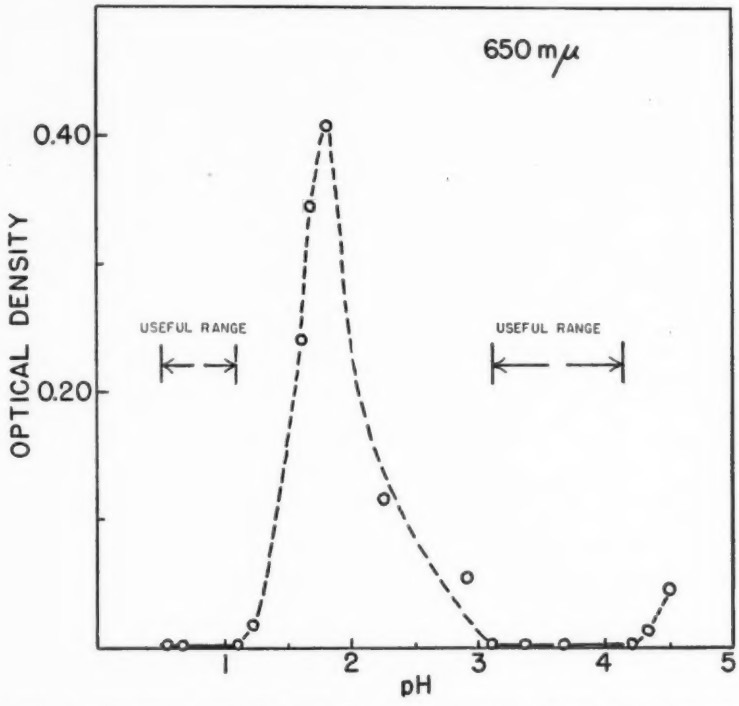

FIG. 3. *Color development in the reagent blank at various levels of pH, showing the range where molybdic acid is itself reduced (650 mμ).*

reduction of phosphomolybdic and molybdic acid, while the former indicates a separation of the two processes, phosphomolybdic acid being the only complex reduced. The data confirm the observations of Lowry and Lopez (10) as to the effect of pH. Of the two useful pH ranges for phosphorus determination, the higher one (pH 3.1 to pH 4.2) is chosen for two reasons. Firstly, the higher pH confers more stability on labile phosphate esters (10) and secondly the wide range confers a greater acid tolerance upon the method. A sodium acetate – acetic acid buffer, pH 4.0 (solution No. 1), has been found to be satisfactory. However in some cases, especially in the estimation of total phosphorus (P_t) after the wet ashing procedure (see later), it has been found more convenient to use a stronger buffer solution made by acidifying 500 ml. of 1*N* sodium acetate with about 110 ml. of glacial acetic acid to pH 4.0.

Effect of the Concentration of Ammonium Molybdate and Ascorbic Acid on the Reduction of Phosphomolybdic Acid

Lowry and Lopez (10) found that the lowest permissible molybdate concentration for full color development was 0.05%. It is the present author's opinion that this concentration verges precariously on the lower safety limits for full color development. The optical density of phosphate solutions containing various concentrations of ammonium molybdate has been plotted against molybdate concentration compared to the reagent blank (0.25% ammonium molybdate) set at zero (Fig. 4). Maximum color development occurs at a concentration of ammonium molybdate over 0.125%; thereafter a

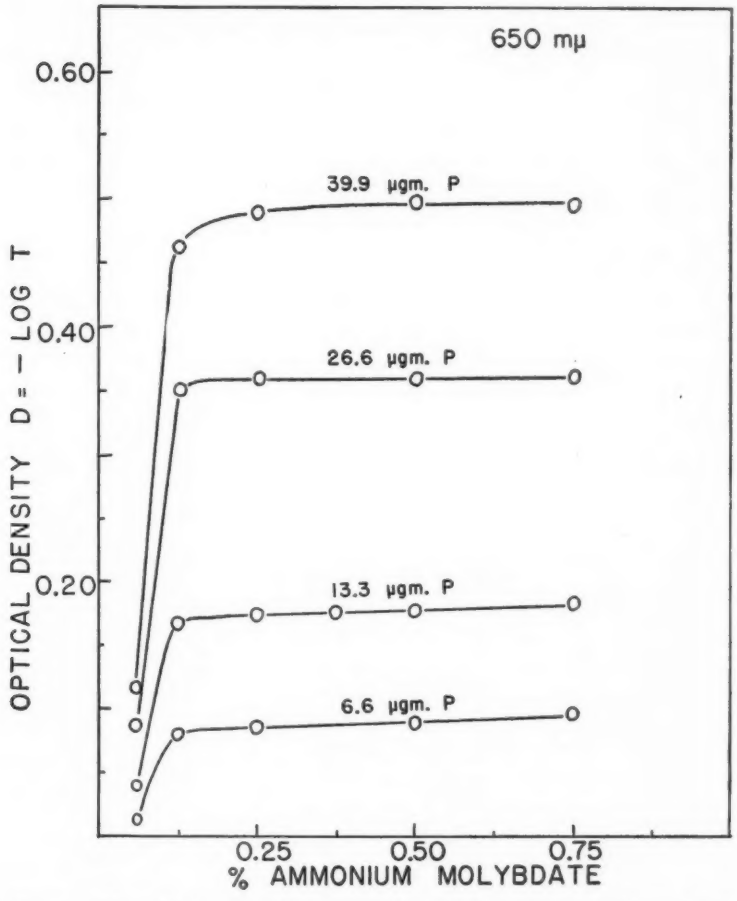

FIG. 4. *Effect of the concentration of ammonium molybdate on color development at four levels of phosphorus (650 mμ, pH 4.0).*

slight increase occurs in the two lower concentrations of phosphorus. At a level of 39.9 μgm. P maximum color development occurs beyond a concentration of 0.25% ammonium molybdate and may account for the deviation from Beer's law at this concentration of phosphorus (Fig. 1). A value of 0.25% ammonium molybdate in the final volume was chosen as the optimum concentration since it is safely past the limiting values of ammonium molybdate for full color development. This concentration is equivalent to 0.5 ml. of 5% molybdate in 10 ml. of the final volume. If difficulties are encountered in the splitting of labile phosphate esters, e.g. acetyl phosphate, at this concentration of ammonium molybdate then a decrease to 0.125% would be permissible providing the concentration of phosphate is kept low.

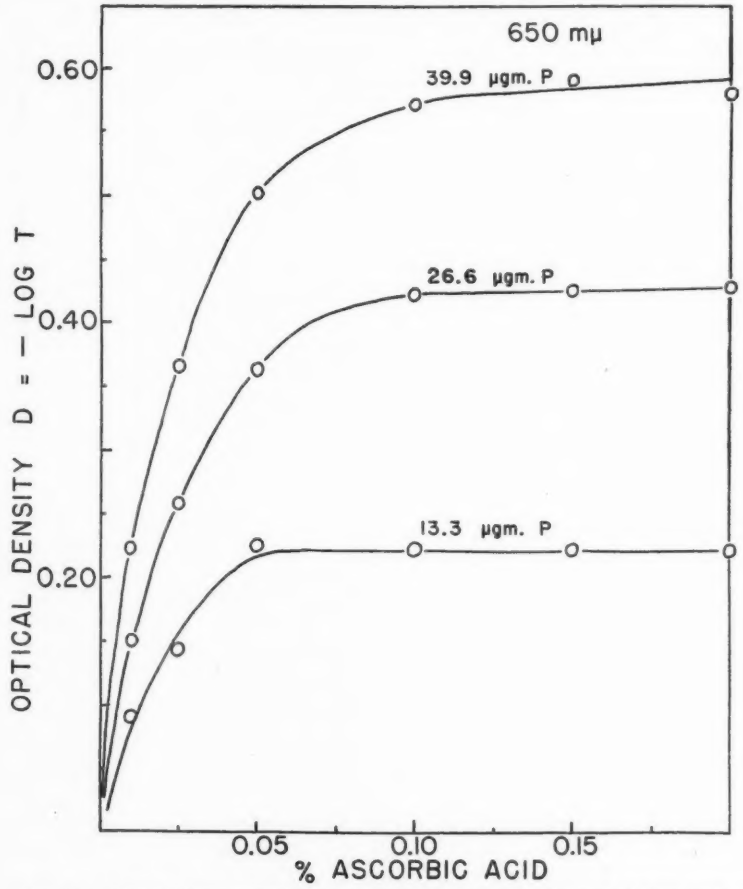

FIG. 5. *Effect of the concentration of ascorbic acid on color development at three levels of phosphorus (650 mμ, pH 4.0.)*

The ascorbic acid functions to reduce the phosphomolybdic acid complex to what is called "molybdenum blue". Color development in phosphate solutions at various concentrations of ascorbic acid in the final 10 ml. volume has been plotted against ascorbic acid concentration in Fig. 5. Maximum color development occurs in the two lower concentrations of phosphorus at 0.10%, thereafter no significant increase is brought about by increasing the ascorbic acid concentration. The highest concentration of phosphate is outside the range of the method. In this experiment the transmittance of each solution was compared against a distilled water blank set at 100% transmittance, thus allowing each curve to be extrapolated to the common graphical origin. A concentration of 0.10%, equivalent to 1 ml. of 1% ascorbic acid in 10 ml. of the final volume, was chosen as the optimum concentration required for the method.

Rate of Reduction of Phosphomolybdic Acid

Prior to ascorbic acid being used as a reducing agent in this reaction the time taken for complete reduction and uniform color development was of the order 30 to 60 min. and even then color instability was observed (5, 7). With ascorbic acid as the reducing agent, under the conditions prescribed, the reaction is complete in approximately five minutes, but the most outstanding feature is the high degree of color stability. Progress curves of the reaction are shown in Fig. 6 at three levels of phosphorus of which the highest is outside the range of the method and has been included as a study of an extreme case. The lowest level of phosphorus (10 μgm. P) in this case was compared against the reagent blank, while the other two (26.6 μgm. and 39.9 μgm. P) were compared against distilled water set at zero optical density, hence the differing points of origin of these curves. Readings were taken for one hour, but have not been included owing to the insignificant variation from the equilibrium state achieved at three to six and one-half minutes at the three levels of phosphorus.

Earlier experiments showed that ammonium molybdate, rather than retarding the rate of color development (10), increased it, and some data on this point, using the Klett-Summerson photoelectric colorimeter, are assembled in Table I.

At the concentration of 0.25% ammonium molybdate, completion of the reaction was observed in approximately three-quarters of the time taken for a solution containing 0.5% ammonium molybdate and one-half the quantity of phosphorus. Fig. 6 shows that with increasing phosphate concentration under standard conditions the time for completion of the reaction is increased. Therefore one must conclude from the data in Table I that sufficiently high concentrations of ammonium molybdate depress the rate of reaction, although not affecting ultimate color development (cf. Fig. 4). This is further justification for maintaining the concentration of ammonium molybdate as low as permissible. Furthermore the presence of excess molybdate provides corresponding opportunity for its reduction to molybdenum blue.

The Interference Due to Silicates

Both silicate and arsenate give rise to heteropoly compounds with molybdates which can be reduced to molybdenum blue (13). The latter would rarely be a significant interfering substance in plant extracts. However those plants

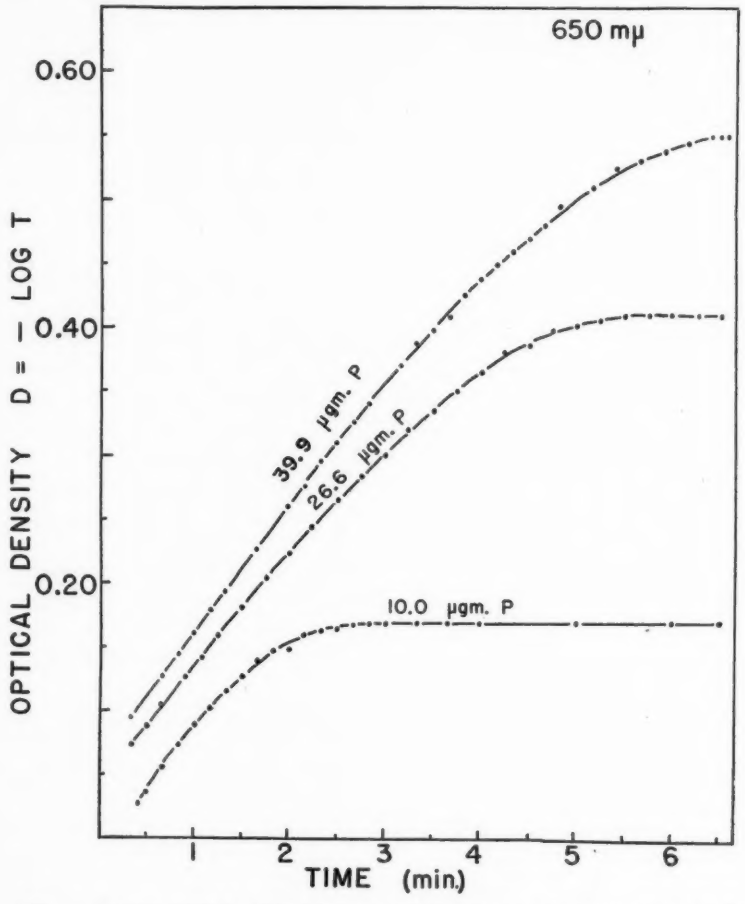

FIG. 6. *Progress curves of the reduction of phosphomolybdic acid by ascorbic acid at pH 4.0 and 650 mμ, showing the rapid rate of reaction and uniform color developed.*

having a liberal silicate economy might possibly present a serious problem in the determination of phosphorus. Woods and Mellon (13) using chlorostannous acid allow a permissible amount of 10 p.p.m. silicon (10 μgm. per ml.) to give an error within 2% phosphorus. A solution of sodium silicate ($Na_2SiO_3 \cdot 9H_2O$) was made up containing 240 μgm. Si per ml. Of this solution

TABLE I

EFFECT OF CONCENTRATION OF AMMONIUM MOLYBDATE ON THE RATE OF REDUCTION OF PHOSPHOMOLYBDIC ACID

10 μgm. phosphorus, 0.5% molybdate		20 μgm. phosphorus, 0.25% molybdate	
Time, min.	Apparent P, μgm.	Time, min.	Apparent P, μgm.
0.75	2.2	0.33	2.1
1.00	4.1	0.58	3.9
1.50	5.8	0.75	6.0
1.66	6.8	1.00	9.9
1.92	7.8	1.33	11.7
2.25	8.5	1.58	13.4
2.75	9.3	1.75	15.2
3.00	9.7	1.92	17.5
3.25	9.8	2.01	19.0
4.00	9.9	2.25	20.5
4.25	9.9	2.33	20.6
4.50	9.9	2.40	20.7
5.00	9.9	3.00	20.7

0.5 ml. (120 μgm. Si) was treated as in the procedure for phosphorus determination and the absorption spectrum followed over the wave length range as shown in Fig. 2. The absorption spectrum almost parallels that of phosphomolybdic acid. If the color development conforms to Beer's law at 650 mμ then approximately 1/10th (cf. Woods and Mellon (13)) of this concentration (i.e. 12 μgm. Si) would give an error within 2% in a total of 10 μgm. phosphorus. Using the normal dilutions for the determination of phosphate in plant extracts, a transmittance of the order produced by 120 μgm. Si would be equivalent to 0.6 gm. per 100 ml. of extract and the permissible amount would be 1/10th or 0.06 gm. per 100 ml. of extract. Latshaw and Miller (9) found a concentration of 2.5% silicon in the dry matter of whole corn leaves. Assuming 90% water content this value would be equivalent to 0.25 gm. per 100 gm. fresh leaves, a value four times as great as that permissible. However it is generally agreed that silicon impregnates the cell walls of plants. Hence procedures that extract juices and the more active metabolic constituents, eliminating cell wall material, would be only slightly affected by the presence of this element. In so far as procedures in this laboratory are concerned, the cell wall material is completely removed by coarse filtering and centrifugation and it is assumed that soluble silicates are not in sufficient proportion to interfere seriously with phosphate determinations in these isolated systems.

THE METHOD ON PLANT EXTRACTS AND ITS APPLICATION TO THE DETERMINATION OF ORGANIC PHOSPHATE COMBINATIONS

Having prescribed the conditions for the estimation of inorganic phosphorus in pure solutions, the method is now considered as to its suitability for the estimation of inorganic and organic combinations of phosphorus in plant extracts. Determinations of inorganic and organic phosphorus may be made

on the fresh or deproteinized juice. In the latter case ice cold 7.5% trichloracetic acid is added to the juice in the proportion 5 : 1. The centrifuged extract is then brought to pH 4.0 by 1*N* sodium acetate and stored in the refrigerator. This procedure results in a solution that requires no further clarification for phosphorus determination.

If investigations are to be made on fresh juice, the juice is filtered through cheesecloth and centrifuged for a few minutes to remove cell debris and other readily precipitated material. The extract may be diluted with water or an appropriate phosphate or other buffer. In this manner an artificial phosphorus nutrition may be set up. The extract at this stage contains material, mostly chlorophyllous and some proteinaceous, that will separate on prolonged, centrifugation, or standing in the cold. If it is desired to separate this material a slight lowering of the pH, e.g. in wheat from pH 5.6 to 4.5, will precipitate such materials readily. The extract now contains proteins, inorganic phosphate, and a small amount of organic phosphate in labile combination, which declines to insignificance if allowed to stand without deproteinization. Fresh extracts are deproteinized at the time of determination.

Inorganic Phosphorus (P_i)

Transfer an aliquot, usually 1 ml., of the extract containing about 500 μgm. P per ml. to a 50 ml. volumetric flask. Deproteinize with 5 ml. of cold 7.5% trichloracetic acid and immediately bring the extract to pH 4.0 by an addition of 1*N* sodium acetate. Make to volume with distilled water, centrifuge for five minutes, and use 1 ml. for the estimation of phosphorus described previously for pure solutions. Recovery tests are shown in Table II.

TABLE II

RECOVERY OF INORGANIC PHOSPHATE FROM WHEAT LEAF EXTRACT

Test material	P_i				P_t			
	μgm./ml.		%		μgm./ml.		%	
	Calc.	Det'd.	Error	Recovery	Calc.	Det'd.	Error	Recovery
1. Wheat leaf extract		310				320		
2. 0.1*M* sucrose, 0.033*M* KH_2PO_4	1035	1100*	5.9		1035	1075*	3.7	
3. Equal parts No. 1 and No. 2	705	700	0.7	99.0	710	700	1.4	100.1
	705	695	1.4	98.0	710	700	1.4	100.1

* *Difference between P_i and P_t in No. 2 = 2.2%.*

Phosphorus in Organic Combinations

Organic phosphates can be distinguished from one another by the differing solubilities of their barium salts (12) and also by the lability of their phosphate

groups when undergoing acid hydrolysis (6). The latter method has been employed in this laboratory whenever it has been unnecessary to identify the compounds as to species, but rather as to type. The procedure using a final concentration of 1*N* hydrochloric acid to effect hydrolysis (6) has been followed, separating the fractions into P_7, P_{60}, and P_{180}, the subscripts referring to the time of hydrolysis in minutes. Total phosphorus (P_t) is estimated by the wet ashing procedure using 60% perchloric acid. All these procedures depend on the liberation of inorganic phosphate groups during hydrolysis and the amount liberated is estimated by the difference between the inorganic phosphorus content of the original extract and the amount present after hydrolysis. For a discussion of the identifiable constituents of the various fractions, the reader is referred to Heard (6).

Seven Minute Phosphorus (P_7)

Transfer an aliquot, usually 1 ml., of the extract containing about 500 μgm. P to a large boiling tube of the type used in sugar determinations. Add 4 ml. of distilled water and 5 ml. of 2*N* hydrochloric acid. Place the tube covered with a glass bulb into a boiling water bath. After seven minutes, cool rapidly and bring to pH 4.0 by the addition of 11 ml. of 1*N* sodium acetate. Transfer the contents of the tube quantitatively to a 50 ml. volumetric flask and make to volume with distilled water. A blank of distilled water is committed to the same procedure. Centrifuge, and use 1 ml. for the determination of phosphorus. The increase in inorganic phosphorus, if any, over the value determined on the extract prior to hydrolysis is called seven minute hydrolyzable phosphate. Table IV shows the procedure adopted to test the validity of the method on a sample of glucose-1-phosphate, an ester that liberates its phosphoric acid group under these conditions of hydrolysis.

Sixty Minute and 180 Min. Phosphorus (P_{60} *and* P_{180})

The procedure is essentially the same as for the seven minute phosphorus determination, except that the tubes are retained in the boiling water bath for their respective times. Each tube in this case is fitted with an air condenser consisting of a long glass tube (length, 55 cm.; inside diameter, 6 mm.) attached to the tube by means of a rubber stopper. Heard (6) found difficulty in obtaining consistent results unless the tubes were sealed. However, the present author has not encountered this difficulty and consistent results have been obtained by this method. Recovery tests for inorganic phosphate are shown in Table III.

Total Phosphorus (P_t)

Total phosphorus is determined by the wet ashing usually of 1 to 2 ml. of the initial extract containing about 500 μgm. P, with 5 ml. of 60% perchloric acid in a small Kjeldahl flask. The flask and contents are heated in a slanting position over a small flame in a fume cupboard until the solution becomes colorless. A blank with distilled water is made at the same time. After cooling, the contents of the flask are washed quantitatively into a 50 ml. volumetric flask with 40 ml. of 1*N* sodium acetate and made to volume.

One ml. is used for the determination of inorganic phosphorus. The difference between this value and the value for inorganic phosphate in the original extract is considered to be total phosphorus in organic combination and is signified by P_u ($P_u = P_t - P_i$). Parts of the P_u fraction may be identified in the P_7, P_{60}, and P_{180} fractions.

Neutralization of the perchloric acid with a 2*N* solution of sodium hydroxide invariably gave high blank values, even if the alkali was stored in a paraffin-waxed container. This was assumed to be due to a continual dissolution of silica from the glass vessel, since the absorption spectra of such blanks were similar to that of the silica complex shown in Fig. 2, and freshly prepared solutions of alkali do not show this characteristic absorption. Although accurate results could be obtained if a standard amount of the alkali was added to the blank and the unknown each time, the use of sodium hydroxide was discontinued owing to this interference, and replaced by 1*N* sodium acetate. It was often more convenient to use the stronger buffer solution described previously.

Recovery Tests of Inorganic and Organic Phosphates on Plant Extracts

A Klett-Summerson photoelectric colorimeter was used in the recovery tests on plant extracts. Known amounts of potassium dihydrogen phosphate, and glucose-1-phosphate were added to extracts of wheat leaves. The results are shown in Tables II, III, IV, and V.

TABLE III

RECOVERY OF INORGANIC PHOSPHATE IN THE 180-MIN. PROCEDURE ON WHEAT SEEDLING EXTRACTS

Test material	$P_i + P_{180}$			
	μgm./ml.		%	
	Calc.	Det'd.	Error	Recovery
1. Wheat seedling extract		50.0		
2. No. 1 + 10 μgm. P	60.0	58.33 60.0	2.8 0.0	83.33 100.0
3. Wheat seedling extract		45.3		
4. No. 3 + 20 μgm. P	65.3	66.0	1.2	103.5

At first inspection it may appear that some of the recoveries especially the recovery of glucose-1-phosphate from wheat leaf extract as shown in Table V are too low or too high. However, it must be remembered that the absolute error of a method is involved in the percentage recovery. If the substance to be recovered is small in proportion to the whole, then a small percentage error will be manifested as an abnormally large deviation in percentage recovery. Thus, percentage recovery cannot be used alone as a criterion of validity, but

TABLE IV

LIBERATION OF PHOSPHATE FROM GLUCOSE-1-PHOSPHATE IN THE 7-MIN., 180-MIN., AND TOTAL PHOSPHATE PROCEDURES

Procedure	Apparent P	Recovery
	μgm./ml.	%
1. 7-min. dist. water blank	0.0 (set)	
2. 7-min. + G-1-P* (room temp.)	1.0	
3. 7-min. + G-1-P*	15.8	100.5
4. 180-min. + G-1-P*	15.6	99.4
5. Total phosphate + G-1-P (wet ashing)*	16.0	101.8

* *Glucose-1-phosphate solution equivalent to 15.7 μgm. per ml. P.*

TABLE V

RECOVERY OF GLUCOSE-1-PHOSPHATE FROM WHEAT LEAF EXTRACT

Test material	P_i			$P_i + P_7$				P_t			
	μgm./ml.		%	μgm./ml.		%		μgm./ml.		%	
	Calc.	Det'd.	Error	Calc.	Det'd.	Error	Recovery	Calc.	Det'd.	Error	Recovery
1. Wheat leaf extract		355							355		
2. G-1-P solution					15.7						
3. Equal parts No. 1 and No. 2	177.5	175.0	1.5	185.3	183.5	1.0	75.0 (on calc.) 99.5 (on det'd.)	185.3	185.0	0.2	96.0 (on calc.) 128.0 (on det'd.)

must be considered together with the percentage error either between determinations or between the theoretical and the experimental. In Table V, percentage recovery has been based on both the theoretical and the experimental value.

The greatest percentage errors are to be found in the difference between the calculated and experimentally determined values for phosphate in the phosphate buffer (Table II). However this is offset by the considerably lower error between phosphate determined by the normal and the total phosphate procedures and, furthermore, by the good recovery obtained from the wheat leaf extracts. On the whole the absolute error of the method lies within 2% and the results shown in Tables II to V inclusive are indicative of the validity of the procedures.

Phosphate Fractions in Wheat Leaves

One of the best methods by which the validity of a procedure is tested is to prepare a balance sheet of the substance under determination. Accordingly, data of this nature are presented here.

Wheat leaves of the Khapli variety, three weeks old, were harvested and passed through a mechanical juicer to separate the major part of the cell wall material. The juice was then filtered through cheesecloth and centrifuged for a few minutes to remove cell debris and initial determinations were made of the inorganic and total phosphate content of the juice. To 100 ml. of extract, 1.5 ml. of sodium acetate (1*N*) – acetic acid buffer pH 4.0 was added to lower the pH from the initial value of pH 5.61 to pH 4.5, at which pH value the colloidal suspension is readily precipitated. The extract was separated into residue and supernatant by centrifuging for about 10 min. and inorganic and total phosphorus determinations were made on 90 ml. of the supernatant, and on the residue made to a total volume of 80 ml. with distilled water. A phosphate balance sheet has been prepared, calculated on the basis of absolute quantities present (Table VI) and this also has been diagrammatically represented in the form of a histogram in Fig. 7.

TABLE VI

BALANCE SHEET OF PHOSPHATES IN WHEAT LEAF JUICE

Fraction of juice	P_t		P_i		$P_u(P_t - P_i)$	
	Mgm.	% of total	Mgm.	% of total	Mgm.	% of total
Whole juice (W)	46.5	100	34.25	73.5	12.25	26.5
Supernatant (S)	33.75	72.6	35.33	76.0	−1.58	0
Residue (R)	12.40	26.7	6.44	13.8	5.96	12.8
S + R	46.15		41.77		4.38	
S + R − W	−0.35		7.52*		−7.87*	

* *Increase of P_i fraction equivalent to decrease in P_u fraction.*

Phosphorus in organic combination is designated as P_u calculated from the difference between P_t and P_i. Whole juice in this case contains 26.3% P_u. and analysis of the supernatant after separation indicates that no P_u is present in this fraction. Therefore it should all be found in the residue, but analysis shows that only half the amount present in the original juice can be recovered in the residue (12.8%). Since the P_i in the supernatant (72.6%) accounts for all of the P_i present in the original juice (73.6%), none of this fraction should be present in the residue, but this is not the case and P_i is found in the residue approximately equivalent (13.8%) to the amount of P_u apparently lost from the residue. This juice was not deproteinized initially and hence changes are to be expected in the various fractions. It appears that the changes occur in the residue after dispersion in water and they are associated with a gradual release of inorganic phosphate (P_i) from the organic phosphates (P_u) probably due to the action of a biological system. This experiment, of course, does not indicate that the P_u fraction is confined solely to the residue. Similar phosphorus cleavage processes may have been occurring in the supernatant during the extraction procedure and subsequent treatment. In fact,

if juice is deproteinized immediately after extraction, organic phosphate fractions are revealed in the supernatant as shown in extract No. 2, Fig. 7. However, apart from this incidental physiological interest the data bear witness to the precision of the method, which audits the balance sheet of phosphates within permissible experimental error.

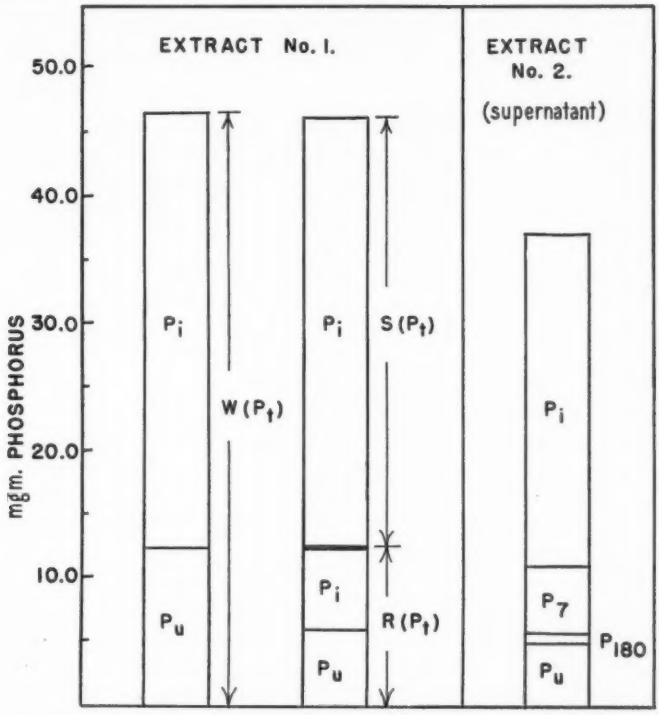

FIG. 7. *Extract No. 1. Phosphate fractions in the whole juice, supernatant, and residue of wheat leaves; W = whole juice, S = supernatant, R = residue. Extract No. 2. A supernatant of wheat leaf juice revealing the presence of organic phosphate fractions.*

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STUDIES ON THE MICROFLORA OF FLOUR[1]

By Norman James[2] and K. N. Smith[3]

Abstract

Bacteria and fungi, each cultured on appropriate media at 25° C., were found to constitute the major groups of organisms in five brands of commercial flour. Numbers of these organisms showed the same trend in relation to brands and the difference between brands was found to be significant. In addition, these brands harbored mesophilic acid-producing bacteria, thermophilic flat-sour spores, anaerobic thermophilic spores, spores of rope-producing bacteria, and yeasts. Numbers of organisms in the latter groups did not show a consistent relationship to brands. The usual method for determining spores of rope-producing bacteria proved to be inadequate for obtaining counts of spores of species known to produce rope in bread. Replication of sampling and careful laboratory procedures were found to be necessary for obtaining reliable estimates of any population in flour.

Wheat flour is the basic food for a large part of the world's population. Any factor that affects its quality is of economic importance to the farmer and the milling industry. Relatively little is known about the microflora of Canadian flour, although it might be expected that flour would harbor soil, dust, and grain types of bacteria, yeasts, and fungi that could affect the quality of products made from it. It would appear logical, therefore, that fundamental information on the flora of normal flour should be available as a background for studies involving specific defects that from time to time confront the milling and baking industries. This study was undertaken to provide some of this basic information.

Historical

Tanner (9) reviewed the literature on the microbiology of flour. Early investigations established certain general facts. Flour harbored large and varying numbers of microorganisms, the difference in numbers being related to differences in moisture and in quality. Certain species of bacteria and of fungi were commonly encountered. Such defects as rancidity and mustiness in flour and ropiness in bread were associated with specific types of organism. Kent-Jones and Amos (8) outlined a procedure for obtaining counts of total bacteria and of spores of rope-producing bacteria in flour. These investigators reported that the washing of grain before milling caused a substantial reduction of bacteria in flour; that patent flours harbored relatively small numbers of bacteria; and that spores of rope-producing bacteria were present in most samples examined. Gustafson and Parfitt (4), using the technique of Kent-Jones and Amos, found that the development of rancidity during

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storage of flour was not related to changes in numbers of bacteria. Two years later Holtman (5), investigating the problem of rancidity, reported that overbleaching tended to increase microbial oxidation of flour. Barton-Wright (2) attempted to find a relationship between bacteria and fungi and chemical changes during storage. He attributed mustiness of flour to fungi and not to bacteria. Of the fungi, 90% belonged to the genus *Penicillium*. Amos (1) found a species of *Streptococcus* and a torula in most samples of flour examined. He isolated from flour *Micrococcus ureae*, *M. liquefaciens*, and *Flavobacterium* sp. This investigator considered the last named to be epiphytic on wheat. James *et al.* (7), in an investigation on the microflora of stored wheat, reported that two types of bacterium, considered by them to be epiphytes, persisted on wheat even after repeated vigorous washings. They stated that the role of these bacteria in determining the properties of cereal products had not been established. Christensen (3) reported on a study on technique for the determination of molds in flour. This investigator found that molds were distributed uniformly in flour. A significantly higher count was obtained when a binocular microscope was used as an aid in counting numbers developing on plates.

Preliminary Studies

The first study was designed to determine the effect of (*a*) time of shaking the initial dilution, (*b*) settling after shaking, and (*c*) difference in medium on counts of fungi in flour. A 10 gm. sample of flour diluted to 0.1 in sterile water and fine gravel was shaken for two minutes on a mechanical shaker. Immediately afterwards a 10 ml. aliquot was pipetted off to make a 0.01 dilution. The initial dilution was reshaken for 28 min., after which a second 10 ml. aliquot was transferred to make a second 0.01 dilution. The initial dilution then was permitted to settle for 10 min. before a 10 ml. aliquot of the supernatant liquid was used to make a third 0.01 dilution. Ten replicate plates were prepared from each of the three 0.01 dilutions. Czapek's agar, acidified by the addition of 1 ml. 10% lactic acid per 100 ml. of medium, was used in five plates; and malt–salt agar (3) in the remaining five. Incubation was at 25° C. for six days. The experiment was replicated 10 times, each with a different flour. The mean count per plate from 100 plates prepared from dilutions shaken for two minutes was 12.95; the mean count from the same dilutions shaken for 30 min. was 14.83; and the mean count from 100 plates prepared from the supernatant liquid was 2.73. The difference between any pair of the three means was found to be significant. The data were used, in addition, to compare counts on the two media. With Czapek's agar the mean count from 150 plates was 14.11, whereas with malt–salt agar it was 16.40. Again, the difference between means was found to be significant.

A second study was designed to determine the errors of sampling and diluting. Using aseptic technique and a sterile spatula about 100 gm. of flour were removed from a sack and mixed thoroughly by a standard procedure. Four replicate 10 gm. portions were diluted to 0.1 dilutions. These were shaken on a mechanical shaker for 30 min. Then from each, four 10 ml.

aliquots were used to make four replicate 0.01 dilutions. Each of these dilutions was shaken by hand 25 times. Then a 1 ml. aliquot was transferred to each of five plates for culturing in acidified Czapek's agar at 25° C. for six days. The experiment was replicated three times, each time from the same sack of flour. The experiment involved 240 plates, representing 48 dilutions, 12 portions, and three samples. The variation in counts between replicate samples was found to be significant; whereas the variation between replicate portions from one sample and the variation between replicate dilutions prepared from one portion were not significant.

Microorganisms in Commercial Flour

Fifty samples, representing five brands milled by one company, were used. The brands were designated Nos. 1, 2, 3, 4, and 5, respectively, in ascending order of ash content. Duplicate samples of each brand, taken directly from the mill stream into sterile 6 oz. containers, were submitted at biweekly intervals, together with the results of routine analyses for ash, protein, and moisture on each brand at each interval. The bacteriological studies were carried out on the one-day-old samples. The system of mixing and diluting was based on the preliminary findings. Each initial dilution was shaken for 30 min. Each higher dilution was made before appreciable settling could take place. Brands 1 and 2 were raised to the 0.01 dilutions. Brands 3, 4, and 5 were further raised to the 0.001 dilutions. The medium for diluting was sterile water (plus gravel in the initial dilution), and not sterile sodium chloride solution, as used by Kent-Jones and Amos and by Christensen. There appears to be no practical reason for using sodium chloride. It is not used ordinarily for diluting water, milk, or soil for bacteriological counts. The final dilutions were used for preparing triplicate plates for each of:

(*a*) Bacteria on nutrient agar at 25° C. for six days. Many investigators (4, 8) have used 37° C. for this determination. Since under normal conditions the bacterial flora of flour would not be exposed to 37° C., it appeared reasonable to use the lower temperature of incubation.

(*b*) Bacteria on brom-cresol-purple dextrose tryptone agar at 25° C. for six days. This medium is used commonly for bacteria in canned products, sugar, and starch. In this study it was used primarily to obtain counts of acid-producers.

(*c*) Fungi and yeasts on Czapek's medium at 25° C. for six days. This medium has been used widely in soil studies. Many species of fungi can be recognized readily on it.

(*d*) Fungi on malt–salt agar (3) at 25° C. for six days. Counts on this medium were made in order to provide additional data for comparison with counts on Czapek's medium.

After transfers were made for the above plates each 0.01 dilution was heated at 90° C. for 30 min. to destroy vegetating organisms. Each heated dilution was shaken 25 times and used for each of:

(*e*) Thermophilic flat-sour spores on brom-cresol-purple dextrose tryptone agar at 55° C. for 48 hr. One ml. from the heated 0.01 dilution was plated in each of 10 plates. A count of the acid-producers on the 10 plates multiplied by 10 represented the number of thermophilic flat-sour spores per gm. of flour.

(*f*) Thermophilic spores on the plates for (*e*). In this case all the bacterial colonies were counted.

(*g*) Anaerobic thermophilic spores in Bacto-liver broth at 55° C. for 72 hr. Two ml. from the heated 0.01 dilution were added to each of 10 tubes of medium. Acid production in one tube was accepted as evidence of the presence of at least one spore. A count of the tubes showing acid production multiplied by five represented the number of anaerobic thermophilic spores per gm. of flour.

(*h*) Spores of rope bacteria in nutrient broth at 37° C. for 72 hr. (8). One ml. from the heated 0.01 dilution was added to each of 10 tubes of the medium. The presence of a characteristic pellicle was accepted as evidence of at least one spore. A count of the tubes showing pellicle formation multiplied by 10 represented the number of spores of rope bacteria per gm. of flour.

The chemical analyses and the results of this investigation are summarized in Tables I and II.

TABLE I

AVERAGE* ANALYSES OF THE FLOURS STUDIED, %

Ingredient	Brands				
	1	2	3	4	5
Ash	0.324 ±0.006	0.354 ±0.026	0.444 ±0.026	0.532 ±0.072	0.602 ±0.038
Protein	11.3 ±0.7	12.3 ±0.7	13.5 ±0.5	13.5 ±1.1	15.3 ±1.1
Moisture	13.6 ±0.3	14.0 ±0.4	13.7 ±0.1	13.9 ±0.3	13.9 ±0.4

* *Average from five determinations.*

The raw data from plates prepared for bacteria on nutrient agar at 25° C. and for fungi on malt–salt agar at 25° C. were submitted to analysis of variance tests. These two groups represent counts that showed the same trend with brands. The results are shown in Tables III and IV.

Similarly the raw data from plates prepared for fungi on Czapek's and on malt–salt agar were submitted to an analysis of variance test. The results confirmed the finding in the preliminary experiment that the malt–salt agar produced significantly higher counts of fungi.

TABLE II

AVERAGE* NUMBER OF MICROORGANISMS PER GM. IN THE FLOURS STUDIED

	Brands				
	1	2	3	4	5
a	2750	7980	17,630	12,900	19,500
b	2010	6600	13,990	8700	16,600
c	39	555	526	1760	720
d	1150	1040	2260	2180	2850
e	1270	1140	3510	2630	3960
f	54	268	12	47	81
g	22	24	176	63	72
h	47	60	822	133	196
i	1	0	2	1	2
j	49	36	84	66	85

* *Average from 10 samples.*

NOTE: *a* = *Bacteria on nutrient agar at 25° C.*
b = *Bacteria on B.C.P. (brom-cresol-purple) dextrose tryptone agar at 25° C.*
c = *Acid-producers on B.C.P. dextrose tryptone agar at 25° C.*
d = *Fungi on Czapek's agar at 25° C.*
e = *Fungi on malt–salt agar at 25° C.*
f = *Yeasts on Czapek's agar at 25° C.*
g = *Thermophilic flat-sour spores at 55° C.*
h = *Thermophilic spores at 55° C.*
i = *Anaerobic thermophilic spores at 55° C.*
j = *Spores of rope bacteria at 37° C.*

TABLE III

SIGNIFICANCE OF VARIATION IN COUNTS OF BACTERIA ON NUTRIENT AGAR AT 25° C.

Source	D.f.	Mean square	F value	F at 5%
Dates	4	643.40	22.03	2.76
Brands	4	454.85	15.58	2.76
Dates × brands	16	168.44	5.77	
Error	25	29.20		

TABLE IV

SIGNIFICANCE OF VARIATION IN COUNTS OF FUNGI ON MALT–SALT AGAR AT 25° C.

Source	D.f.	Mean square	F value	F at 5%
Dates	4	145.62	16.73	2.76
Brands	4	1632.77	187.67	2.76
Dates × brands	16	197.27	22.67	
Error	25	8.70		

Discussion

The necessity for care at the various sampling steps in the procedure for determining numbers of organisms of any type in flour is obvious when it is considered that the count is made on a mere 0.01 or 0.001 gm. of the flour. This does not imply that emphasis should be placed on unnecessary refinements in technique or that the procedure need be complicated. In the preliminary study on sampling it was found that the variation in counts between replicate dilutions, or that between replicate portions of the mixed sample, was within the limits of experimental error for the method. The basic error of the procedure for obtaining counts by the plating technique had previously been established (6) as the variation between counts from replicate plates prepared from one dilution. This may be accepted as evidence that the procedure for mixing the sample and preparing dilutions was satisfactory. On the contrary, the finding of a significant difference between counts on replicate samples in the preliminary study and between samples of any brand tested on different dates points to the inadequacy of an estimate based on a single sample and to the need for replication of sampling.

Bacteria cultured at 25° C. and fungi cultured at the same temperature evidently constitute the major groups of organisms on freshly milled flours. These groups showed the same trend in relation to brands, and the difference between brands was found to be significant. This trend did not conform to expectancy on the basis of ash content. Brand 3 harbored more bacteria and fungi, cultured at 25° C., than did Brand 4, even though the ash content of Brand 4 was higher than that of Brand 3 on all samples of these brands tested. The protein content of Brand 3 was higher than that of Brand 4 in three of the five samples of each brand studied. The difference in protein content reflects a variation in the materials blended that conceivably might effect a difference in the microbial population of these brands. The data provide little evidence of the superiority of either medium in relation to the other for culturing bacteria at 25° C.; or of either of the media used for culturing fungi at 25° C. The brom-cresol-purple dextrose tryptone agar, even though yielding counts of bacteria about 25% lower, may be preferred because of providing specific information on numbers of acid-producers, in addition to that on total numbers. Similarly Czapek's agar for counts of fungi may be preferred because of its general use in certain studies on soil fungi. It should be recognized, however, that the populations developing on the two media used for bacteria are not identical and that the same holds true on the two media used for fungi. In this study counts of fungi on plates were made with the unaided eye. The use of a binocular microscope, magnification ×10 to ×25, as prescribed by Christensen (3) for counting fungal growths, is open to question. Such magnification is not used in standard procedures for determining numbers of fungi in other products. Further, information is not available to indicate that growths not visible by the unaided eye represent slow growers merely. They may represent second generation growths from species that scatter spores. If so, they should not be counted.

As was found to be the case on wheat samples (7), yeasts in flour appear to vary erratically, with little evidence of consistency in numbers in relation to other types of microorganism, or to ash or protein content. Yeasts and acid-producing bacteria cultured at 25° C. conceivably could affect fermentation during the leavening process.

The finding of spores of thermophilic bacteria (cultured at 55° C.) in flour was not unexpected. They are present in soil and have been isolated from heating composts and manure. They are known to cause certain types of spoilage in canned products. In some cases their presence in the canned product has been attributed to the sugar used. They could withstand the cooking of the baked product and become active after it was removed from the oven and before it was cooled to the normal holding temperature.

The count of spores of rope-producing bacteria by the procedure used, at best, gives only presumptive evidence of the bacteria that could cause ropiness. It is merely a count of heat-resistant types that produce a characteristic pellicle on nutrient broth. Many species produce such a pellicle. In this study 78 pure culture isolates considered tentatively to represent rope bacteria because of pellicle formation were grouped on the basis of sugar fermentations. Of this number only 16 gave sugar reactions that agreed with those of *Bacillus mesentericus* or *Bacillus panis*, two species known to produce ropiness in bread. Of course, on the basis of probability a flour that contains a large number of such pellicle formers would be likely to contain the specific bacteria that produce ropiness. The problem of rope-producing bacteria in flour appears worthy of detailed study under controlled conditions.

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NOTES ON SEED-BORNE FUNGI

VI. SORDARIA[1]

By R. F. Cain[2] and J. W. Groves[3]

Abstract

In the examination of agricultural seeds for the presence of seed-borne fungi, five species of *Sordaria* have been isolated. Three species, *S. fimicola* (Rob.) Ces. & De Not. isolated from a wide variety of seeds, *S. humana* (Fuckel) Winter isolated from corn, and *S. setosa* Winter isolated from beets, are common coprophilous fungi. Two species, *S. inaequalis* Cain isolated from a variety of seeds, and *S. curvispora* Cain from carrot and celery seed are described for the first time. The fungi do not appear to be of any pathological significance.

The Genus *Sordaria*

The genus *Sordaria* was established by Cesati and De Notaris (4) for a miscellaneous assemblage of species of sphaeriaceous fungi. From the time of its origin it has been used by different authors to include various groups of species most of which have a coprophilous habitat. One of the largest of these groups has appendaged spores, that is spores with either primary or secondary appendages or both. The characters used to limit the genus have varied from time to time in the different treatments.

The genus as understood at present includes a number of groups of more or less unrelated species. Various attempts have been made to divide the genus, but most of these divisions have been based on single characters and have resulted in an artificial separation of species showing natural affinities into genera comprised of unrelated forms. For example, *Pleurage* was separated by Griffiths (6) largely on the basis of ascus dehiscence. The asci of many of the species included by him in the genus are perforate at the apex just as in the species included by the same author in *Sordaria*. Likewise, species with asci containing more than eight spores have been grouped in the genus *Philocopra*. This character does not delimit a natural group but has arisen in various lines of evolution in several groups.

The genus *Sordaria* is retained largely on the basis of usage by the present authors in the more comprehensive meaning to include species in which the spores are variously ornamented with a gelatinous sheath, primary appendage or secondary appendage, or various combinations of these. Used in this sense the genus includes various groups of species probably not closely related,

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but detailed knowledge of their morphology and life history is still too inadequate to make an acceptable partition into separate genera, although this will ultimately be advisable.

If it is found desirable to retain the name *Sordaria*, it should be added to the list of genera conservanda and a lectotype chosen. This choice should be made with considerable care in order to avoid future confusion. When first used by Cesati and De Notaris in 1863 (4) 16 species were included although four of these were not transferred from *Sphaeria*. The first of these, *S. sordaria* (Fr.) Ces. & De Not., although somewhat doubtful, is now generally included in the genus *Rosellinia* or a segregate of it. *S. fimeti* (Pers.) Ces. & De Not. is placed in *Hypocopra* by Fries. *S. coprophila* (Fr.) Ces. & De Not., unlike most of those with appendaged spores, belongs to a different group closely related to or included in the genus *Bombardia* Fries. This species should not be chosen as the lectotype of *Sordaria* as was done by Clements and Shear (5).

Sordaria fimiseda Ces. & De Not. is the only other well known species with appendaged spores included by Cesati and De Notaris. If *Sordaria* is to be conserved for a group with appendaged spores, this species should be selected as the lectotype. It should be pointed out, however, that this species is a synonym of *Podospora fimicola* Cesati, which is the type of the genus *Podospora* established by Cesati (3) in 1856 antedating *Sordaria*. To use *Podospora* in the more comprehensive sense will require the transferring to it of a number of species having nonappendaged spores. Furthermore the genus *Podospora* is a synonym of *Schizothecium* established by Corda in Icones Fung. 2:29. 1838.

Sordaria fimicola (Roberge) Ces. & De Not. (not *Podospora fimicola* Ces.) seems to be the more logical choice as a lectotype for *Sordaria*. In this species the spores have a gelatinous sheath instead of appendages. It is included in the group more frequently assigned to *Sordaria* when this genus is used in a more restricted sense.

A number of coprophilous species that are evidently not closely related to either *Sordaria fimicola* (Rob.) Ces. & De Not. or *Sordaria fimiseda* Ces. & De Not. have been included in the genus *Sordaria*. Two of these groups have been segregated by the senior author (2) and transferred to *Coniochaeta* and *Bombardia* respectively.

To recapitulate, the genus *Sordaria* should ultimately be divided into several genera that would include species with natural affinities. As a basis for this division, a more adequate knowledge of the morphology and development of the species is required. An attempt should be made to establish the status of the various generic names now available for these segregated groups.

Some of the species, such as *Sordaria fimicola* (Rob.) Ces. & De Not., which are usually found on dung, frequently occur on other substrata, such as decaying vegetation. It is, therefore, not surprising to find a few species belonging to this genus among a large number of seed samples. The five species that have been encountered belong in three distinct groups. The first

of these includes *S. fimicola* and *S. humana* the spores of which have gelatinous sheaths but no appendages. *S. setosa* belonging to the second group has a primary appendage as well as secondary appendages on the spore. In the third group are two species described as new that show a relationship with *Sordaria curvula* De Bary.

Sordaria fimicola (Rob.) Ces. & De Not., Comm. Soc. Crit. Ital. 1 : 226. 1863. (Fig. 1)

The mycelium of this species produces a rapid and luxuriant growth on malt agar. Numerous pyriform perithecia are soon produced in a superficial layer. These are black when viewed with reflected light but dark brown with transmitted light. The outer wall is bare except for the hyphae that surround the basal part and attach the perithecium to the substratum. There is a single palisadelike layer of cylindrical, eight-spored asci that are attached at the base and surrounded by a layer of hyaline nutritive cells. The spores are obliquely uniseriate, dark brown to nearly black, ellipsoid, rounded at the ends but more acutely below with a circular germ pore at the lower end. A broad, hyaline gelatinous layer surrounds the spore but does not cover the germ pore at the base. The spores produced in cultures are much more variable in size than those grown on dung. In the cultures obtained from seeds they measure 19-27 × 11-15μ but mostly 23-24 × 12-14μ. This is slightly larger than those grown under natural conditions where they are much more uniform in the same perithecium and in different collections, measuring 19-23 × 11.0-12.5μ. A somewhat similar abnormality has been noted in cultures obtained from dung.

Sordaria fimicola has been isolated from seeds of *Apium graveolens* L. var. *dulce* DC. (celery), *Beta vulgaris* L. (mangels), *B. vulgaris* L. var. *cicla* L. (Swiss chard), *Brassica oleracea* L. var. *botrytis* L. (cauliflower), *Capsicum annuum* L. (pepper), *Cucumis sativa* L. (cucumber), *Cucurbita maxima* Duch. (squash), *C. Pepo* L. (pumpkin), *Daucus carota* L. var. *sativa* DC. (carrot), *Festuca rubra* L. (red fescue), *Glycine max* Merr. (soybeans), *Linum usitatissimum* L. (flax), *Lycopersicon esculentum* Mill (tomato), *Pastinaca sativa* L. (parsnip), *Phaseolus vulgaris* L. (beans), *Pisum sativum* L. (peas), *Raphanus sativus* L. (radish), *Spinacia oleracea* L. (spinach), *Taraxacum kok-saghyz* Rod (Russian dandelion), *Trifolium hybridum* L. (alsike), *Triticum aestivum* L. (wheat), *Vicia Faba* L. (broad beans).

This species has been isolated from seeds from Prince Edward Island, Quebec, Ontario, Manitoba, and British Columbia in Canada, from Connecticut and Michigan in the United States, and from Holland and the U.S.S.R.

Sordaria humana (Fuckel) Winter, Botan. Z. 30 : 835. 1872. (Fig. 2)

This species closely resembles *Sordaria fimicola* but can be distinguished by means of the spores, which are distinctly broader in proportion to their length. In the cultures from seeds these measure 22-28 × 16-19 (mostly 23-25 × 16-18 μ). As in the previous species, the spores are more variable in size when grown in culture compared with those produced under natural conditions.

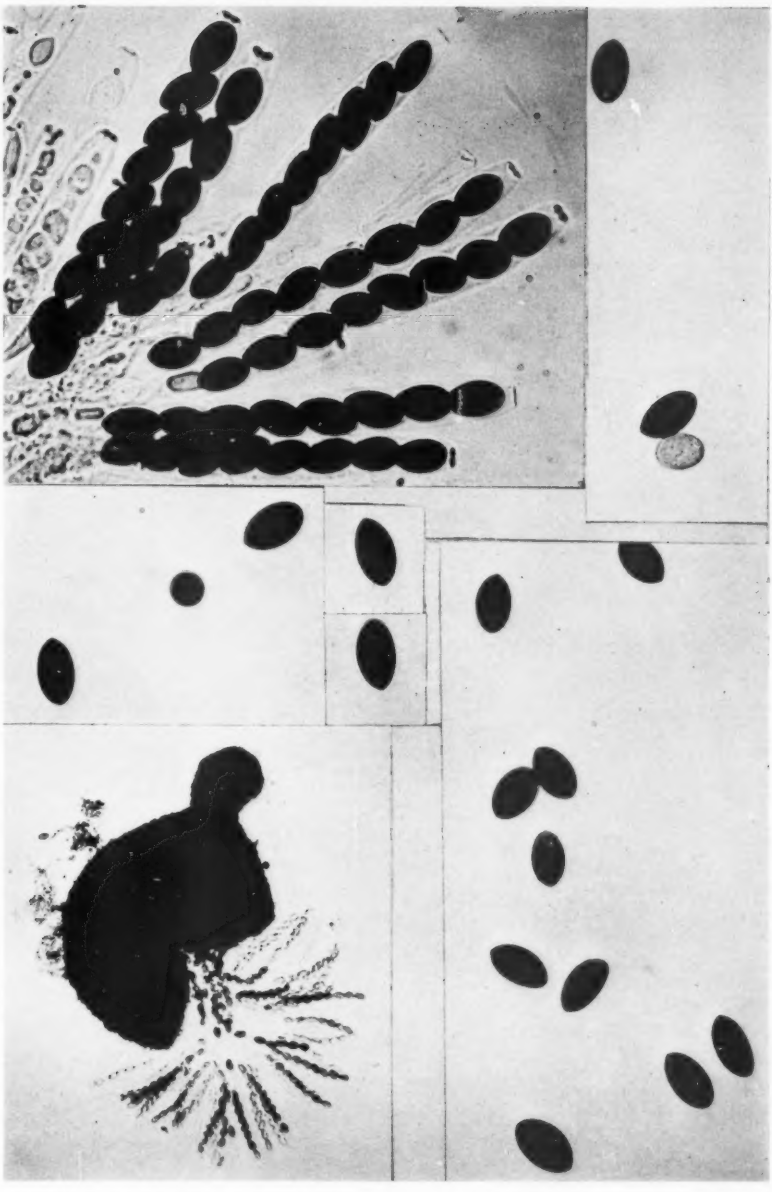

FIG. 1. *Sordaria fimicola. Microphotographs of crushed perithecium, asci, and ascospores. Note the distinct pore in the apex of the ascus. The gelatinous sheath surrounding the ascospores is not evident.*

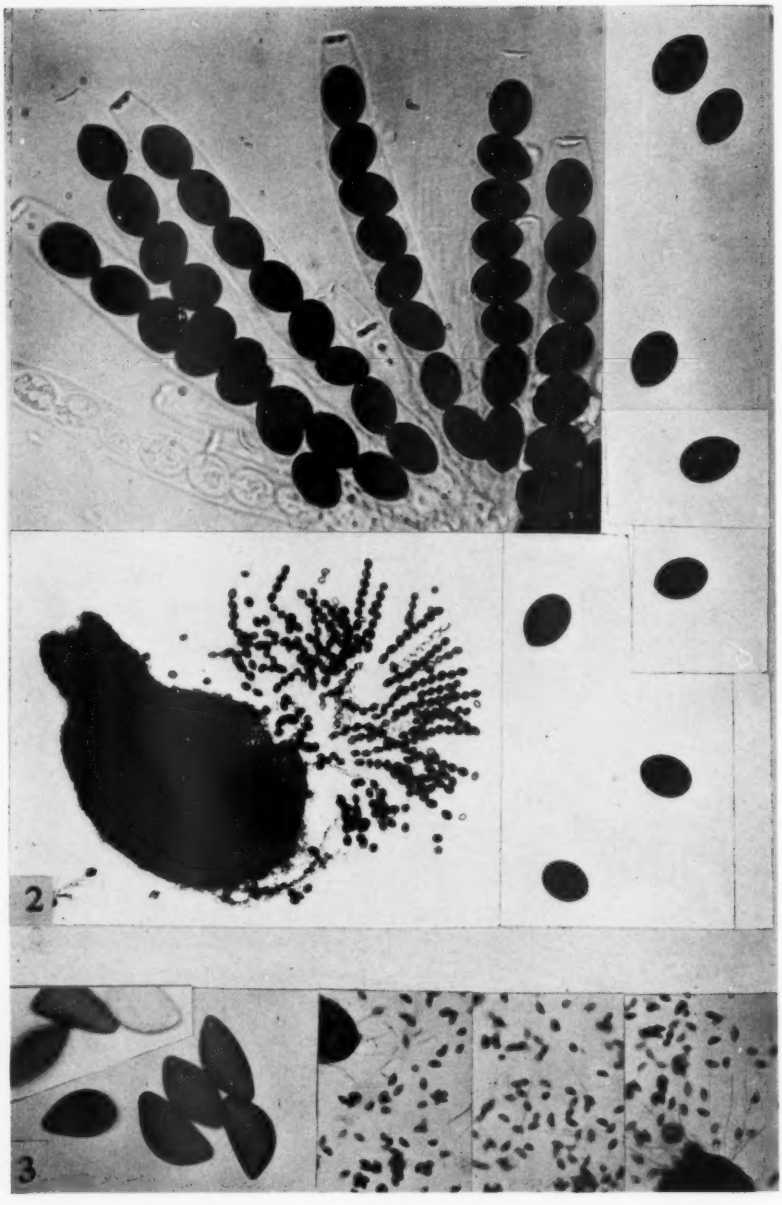

FIG. 2. *S. humana. Microphotographs of crushed perithecium, asci, and ascospores.*
FIG. 3. *S. inaequalis. Microphotographs of ascospores. Note the hyaline, circular germ pore in the spore at the left.*

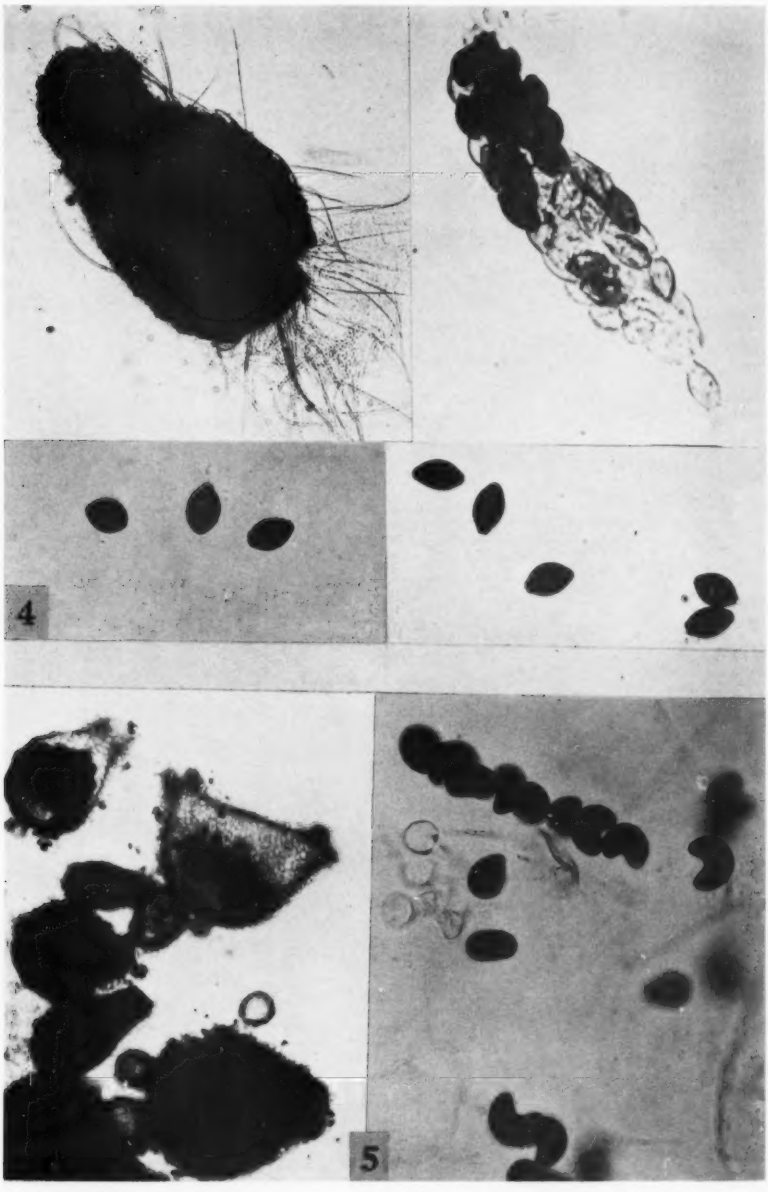

FIG. 4. *S. setosa. Microphotographs of crushed perithecium, showing scattered hairs, ascus with numerous spores, and ascospores.*

FIG. 5. *S. curvispora. Microphotographs of perithecia (one showing two necks and two ostioles), ascus, and ascospores.*

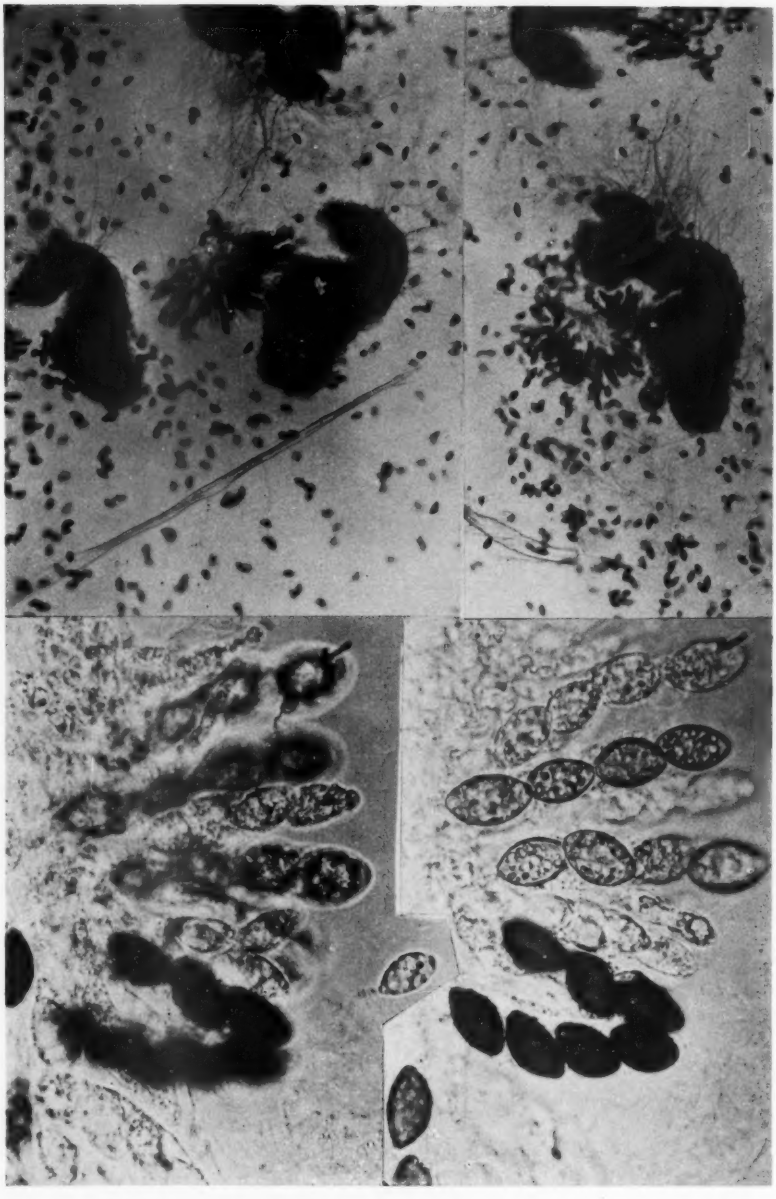

FIG. 6. *S. inaequalis. Microphotogr ıphs of perithecia, with vesicular hair cells covering the outside, four-spored asci, and ascospores. Note the immature, hyaline spore by itself in the central lower half of the plate showing the primary appendage directed to the left.*

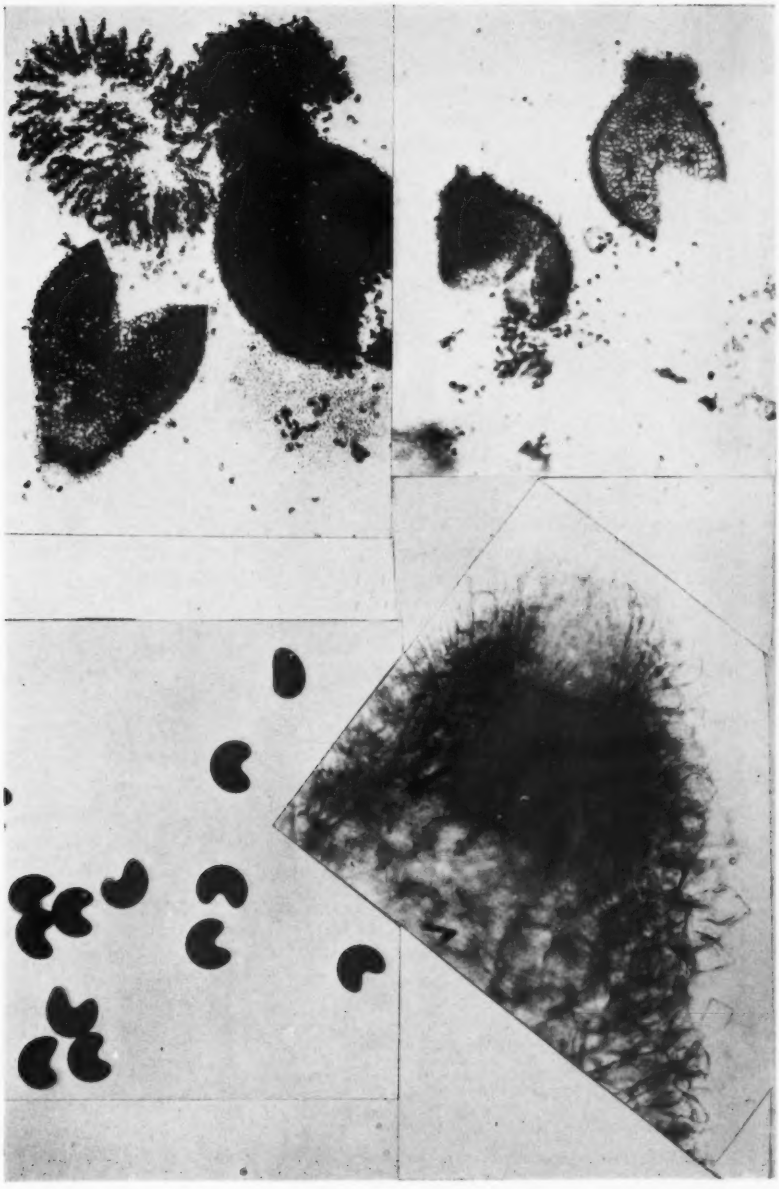

FIG. 7. *S. curvispora. Microphotographs of perithecia, asci, and ascospores. In the lower right is shown a highly magnified upper part of a perithecium showing the neck region with ostiole and the covering layer of fimbriate hair-cells. Note the germ pore in the more highly magnified ascospore near the center of the plate.*

Sordaria humana has been isolated only once from seeds of *Zea Mays* L. (corn) from the Horticulture Division, Central Experimental Farm, Ottawa, Ont.

Sordaria setosa Winter, Abh. Nat. Ges. Halle, 13 : 97. 1873. (Fig. 4)

Cultures of this species isolated from seeds produce numerous pyriform, olivaceous-brown perithecia. The papilliform necks are roughened with minute black papillae. The upper part of the perithecium and neck is covered with scattered, straight, faintly septate, brown hyaline-tipped hairs, which measure 150-250 × 3-4 μ. The lower part has a dense covering of slender hyphae. The asci are fusiform-clavate and contain numerous spores. The ascospores are ellipsoid, dark brown and opaque, measuring 17-21 × 11-13 μ. This size is somewhat smaller than that of the spores produced under natural conditions where they measure 19-24 × 11-16 μ. Each spore has at the base a hyaline cell or primary appendage, which measures about 11 × 3 μ. This cell and the apex of the spore each has a long slender lashlike secondary appendage.

Sordaria setosa has been isolated only once from seeds of *Beta vulgaris* L. (beets) from British Columbia.

Sordaria inaequalis Cain sp. nov. (Figs. 3, 6, 8 to 15)

Peritheciis dense aggregatis, superficialibus, elongato-ovatis, elongato-conicis, interdum subcylindricis, parvis, 240-430 × 110-180 μ, olivaceo-atris; perithecii membrana semitranslucida, tenui, e cellulis magnis, tenui tunicatis, transverse elongatis, 10-25 μ, constituta, cellululis ovatis, conglutinatis, semi-translucidis, olivaceis, tuberculatis, 10-20 μ diam., vestita; collo brevo, crasso, pilis elongatioribus vestito, periphysibus praedito. Ascis tetrasporis, cylindraceis, 90-115 × 11-14 μ, superne late rotundatis, basi in stipitem 15-30 μ longum attenuatis. Sine paraphysibus sed cellulis magnis, hyalinis, vesiculiformibus praeditis. Ascosporis plerumque oblique monostichis, assymetricis, altera parte prope recta, altera valde fornicata, 17-22 × 10-13 μ, initio hyalinis, dein olivaceo-nigris et opacis, basi appendice primaria, clavata, hyalina, 4-8 × 1-2.5 μ, ornatis, appendicibus secondariis destitutis, foramine orbiculato, subapice, praeditis.

Type: In seminibus *Dauci carotae* L. var. *sativae* DC., Minneapolis, Minn., U.S.A., Feb. 8, 1940, TRT *22440*.

Perithecia densely aggregated, superficial, elongate-ovate, elongate-conical, or nearly cylindrical, small, 240-430 × 110-180 μ, olivaceous-black; wall semitransparent, thin, consisting of large thin-walled transversely-elongated cells measuring 10-25 μ in diameter, covered on most of the outside with a layer of ovate, nearly transparent, olivaceous cells measuring 10-20 μ in diameter that usually terminate in one or sometimes two short tubercles; toward the apex of the perithecium these become more slender as well as more densely and uniformly aggregated; neck short, broad, merging imperceptibly into body of perithecium, lined with periphyses. Asci four-spored, cylindrical, 90-115 × 11-14 μ, broadly rounded above, tapering below into a stipe 15-30 μ

long. No distinct paraphyses but with large, hyaline, vesicular, nutritive cells. Ascospores uniseriate, lying obliquely or sometimes parallel, inequilaterally ellipsoid, flattened to almost a straight line on one side, 17-22 × 10-13 μ, hyaline when young, becoming olivaceous and then olivaceous-black and opaque; primary appendage clavate, hyaline, 4-8 × 1-2.5 μ at base of spore; germ pore circular, on side with greatest curvature just below apex.

Type: Isolated in culture from seeds of *Daucus carota* L. var. *sativa* DC. Minneapolis, Minn., U.S.A. Crypt. Herb. Dept. of Botany, University of Toronto *22440*. Additional specimens from same isolation in Herb. Div. Botany and Plant Pathology, Dept. of Agriculture, Ottawa. DAOM *15200* and in Herb. RFC *12049*.

Sordaria inaequalis has been isolated from seeds of *Allium cepa* L. (onion), *Beta vulgaris* L. (beets), *B. vulgaris* var. *cicla* L. (Swiss chard), *Daucus carota* L. var. *sativa* DC. (carrot), *Pastinaca sativa* L. (parsnip), *Petroselinum hortense* Hoffm. (parsley), and *Pisum sativum* L. (peas).

It has been isolated from seeds from Ontario in Canada, and from Connecticut, Pennsylvania, Ohio, Illinois, Minnesota, and California in the United States.

Cultures of this species on malt agar produced rapidly growing, grayish colonies with abundant aerial hyphae forming a fluffy mass. Hyphae 1.5-3.0 μ, sparingly branched, remotely septate, very light brown, forming small strands.

The perithecial initial (Fig. 9) starts as a small inconspicuous coil on a short side branch (Fig. 8). No spermatia were seen.

In some cultures many abnormal types of perithecia were produced as illustrated in Fig. 10. The normal shape is illustrated in Fig. 6. In one instance an abnormal spore with a transverse septum in the dark part was seen (Fig. 15).

This species represents a specialized development from a group of species of which *Sordaria curvula* De Bary is a typical representative. The group includes *S. tetraspora* Wint. (asci four-spored), *S. minuta* Fuckel (asci eight-spored, cylindrical), *S. curvula* De Bary (asci eight-spored, clavate), *S. coronifera* Grove (like *S. curvula* but larger throughout), *S. glutinans* Cain

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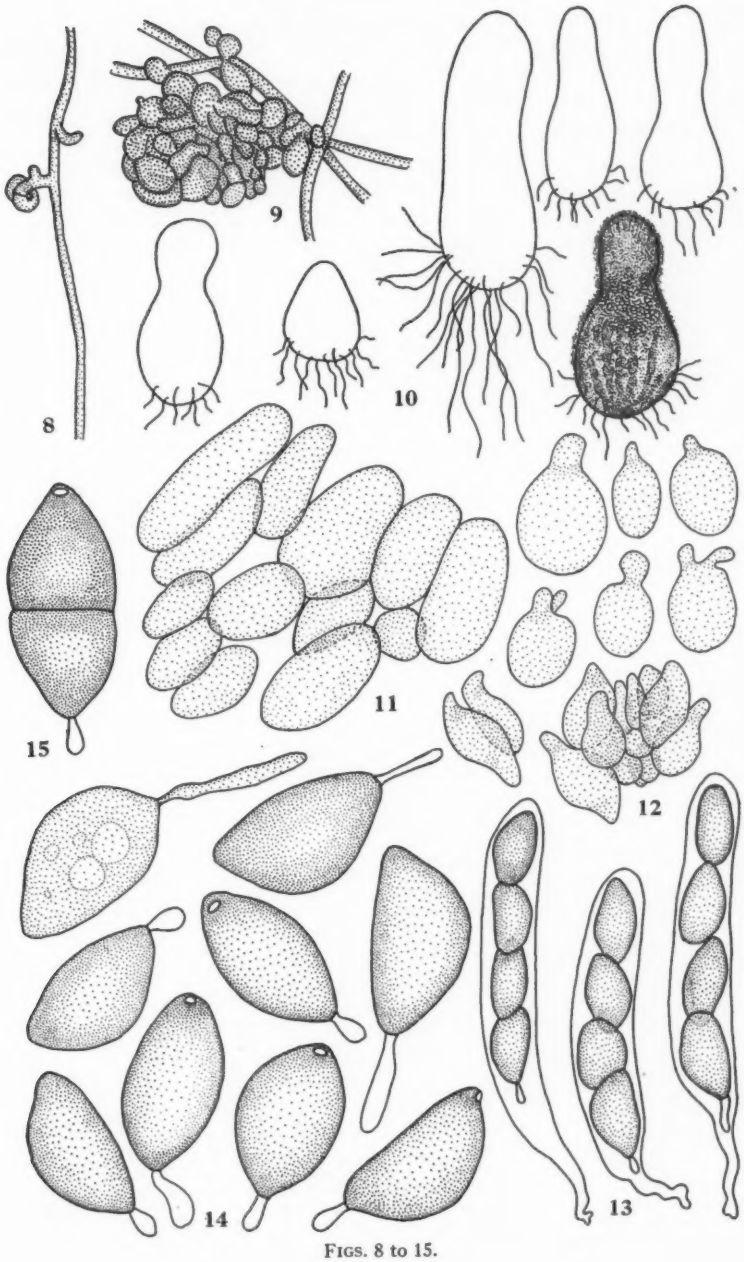
EXPLANATION OF FIGURES

FIGS 8 to 15. *S. inaequalis.* FIG. 8. *Ascogonial coil as a side branch on a hypha. Note the small projection at the base of the coil that will participate in the formation of the wall of the perithecium.* × *960.* FIG. 9. *Primordium of perithecium showing the early development of the wall around the ascogonium.* × *960.* FIG. 10. *Outlines of perithecia showing various shapes, mostly somewhat abnormal. The more normal shape is shown in* FIG. 6. × *90.* Fig. 11. *Cells from outer layer of perithecial wall.* × *1300.* FIG. 12. *Hair cells that cover the perithecium. The lower ones are from the region around the ostiole, the upper from the mid portion of perithecium.* × *1300.* FIG. 13. *Three asci with mature spores.* × *600.* FIG. 14. *Ascospores showing the primary appendage and germ pore. The spore at the upper left is immature and nearly colorless with an abnormally long primary appendage.* × *1300.* FIG. 15. *An abnormal spore with a transverse septum.* × *1300.*



FIGS. 8 to 15.

(with long, flexuous as well as agglutinated hairs), *S. dubia* Hansen (asci 16- to 32-spored), and *S. fimbriata* Bayer (asci eight-spored, spores inequilateral). *S. cervina* Cain and *S. linguiformis* Cain may belong in this series but have a different type of secondary appendage.

Of these species *Sordaria fimbriata* has characters that relate it more closely with *S. inaequalis*. The former has eight instead of four spores in the ascus. The spores are very similar except that they are somewhat larger in the four-spored species as would be expected if this were derived from the eight-spored species. In *S. inaequalis* the perithecia are smaller but otherwise similar, the agglutinated hairs are reduced in size and the end cells somewhat less fimbriate. It seems logical to conclude that this species has been derived from a form very similar to *S. fimbriata* by certain types of specialization.

The latter species, like *S. curvula*, produces spermatia in phialides. Several species having a similar type of spermatial production have been described in the genera *Phialophora* and *Cadophora*. Cultures of *S. inaequalis* have been searched to locate spermatia and phialides but, so far, none have been found. Probably the ascospores contain two nuclei when delimited in the ascus so that the cultures from monospores would be self fertile as in *S. anserina* (Ces.) Wint. The spermatia thus becoming functionless have apparently been lost.

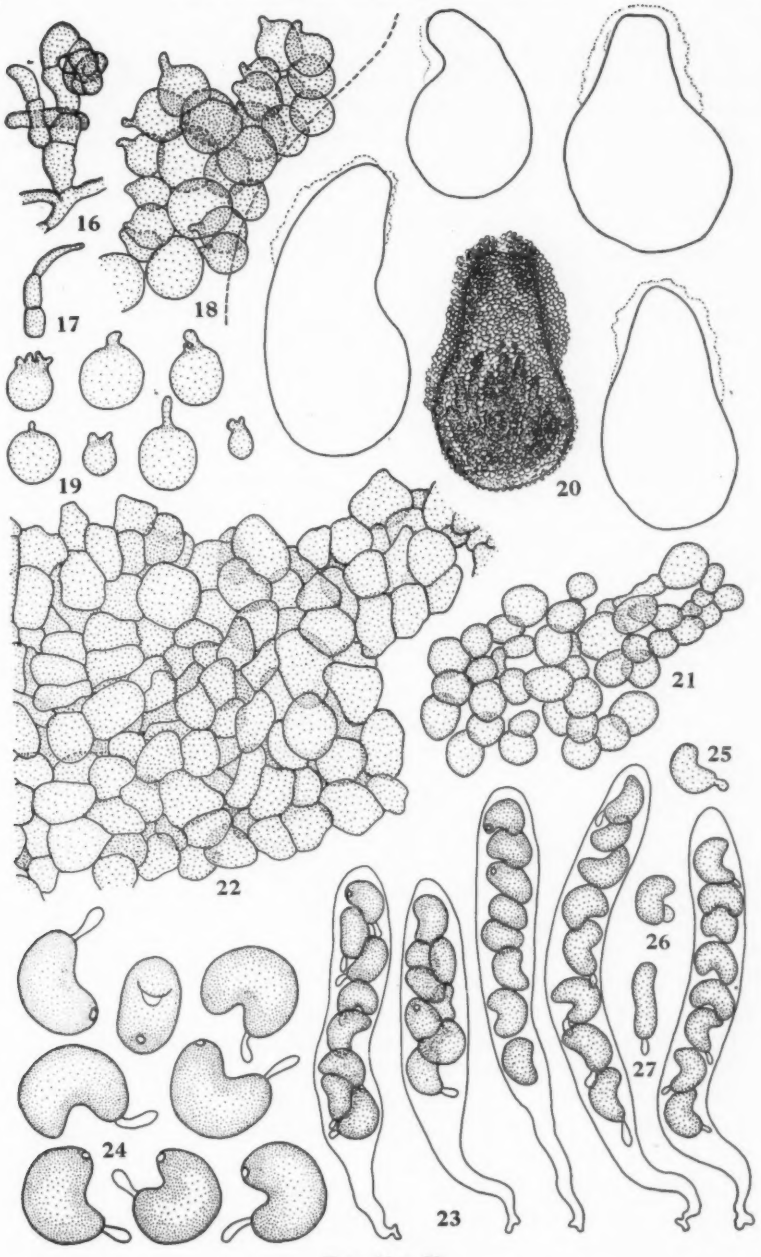
There is nothing in the description of *Bombardia lunata* Zickler (8) to indicate that it is specifically distinct from *Sordaria fimbriata* Bayer (1) so that it should probably be reduced to synonymy under the latter name, which has priority. It is possible that *Sordaria vratislaviensis* A. Schmidt (7, p. 32) is the same species and if so, is the proper name to use since it is older than either of the other two.

Sordaria curvispora Cain sp. nov. (Figs. 5, 7, 16 to 27)

Peritheciis dense aggregatis, superficialibus, elongato-ovatis vel oblongo-conicis, 350-450 × 200-230 μ, olivaceo-atris; perithecii membrana tenui, semitranslucida, pallido-brunnea e cellulis magnis, ventricosis, usque ad 20 μ in diametro, constituta cellulis ovatis, usque ad 15 μ diam., conglutinatis, pallido-brunneis, tuberculatis, vestita; collo brevo, crasso, pilis elongatioribus filiformibus, 0-2-septatis, dense aggregatis vestito, periphysibus praedito.

EXPLANATION OF FIGURES

FIGS 16 to 27. *S. curvispora.* FIG. 16. *Ascogonial coil showing two branches from the basal cells that will participate in the development of the perithecial wall.* × *960.* FIG. 17. *Single hair from the region of the ostiole.* × *840.* FIG. 18. *Agglutinated hair cells forming the outside covering of perithecium taken from the base of the neck.* × *840.* FIG. 19. *Terminal cells of hairs showing the tubercular projections at the apex.* × *840.* FIG. 20. *Five perithecia.* × *90.* FIG. 21. *Agglutinated hair cells covering the perithecium, taken from the mid region.* × 840. FIG. 22. *Cells from outer layer of perithecial wall.* × *540.* FIG. 23. *Five asci with mature spores. The ascus that is second from the left has swollen out after standing in a water mount.* × *840.* FIG. 24. *Ascospores showing primary appendage and germ pore.* × *1300.* FIG. 25. *Ascospore in which a septum has failed to separate the primary appendage so that the entire cavity has become light brown.* × *840.* FIG. 26. *Dark brown ascospore with a light brown appendage separated by a septum.* × *840.* FIG. 27. *An abnormal ascospore that has failed to develop the normal curvature.* × *840.*



FIGS. 16 to 27.

Ascis octosporis, cylindraceis, 100-110 × 11-13 μ, superne late rotundatis, apice obscure perforatis, basi in stipitem brevem attenuatis. Sine paraphysibus sed cellulis magnis, hyalinis, vesiculiformibus praeditis. Ascosporis incomposite monostichis, valde curvatis, altera parte valde impressa, altera valde fornicata, 11-13 × 6-8 μ (interdum minus curvatis et tunc usque ad 17 μ longum), denique fuscis brunneo-atris et opacis; basi appendice primaria, clavata, hyalina, parva, circa 5 × 1 μ, ornatis, appendicibus secondariis destitutis; apice foramine orbiculato praeditis.

Type: Ex seminibus *Dauci carotae* L. var. *sativae* DC., Gilroy, Calif., U.S.A., Feb. 8, 1940. TRT *22439*.

Perithecia forming a dense layer on surface of agar, superficial, elongate-ovate to oblong-conical, 350-450 × 200-230 μ, olivaceous-black, covered with a layer of hairs consisting of a few light-brown globose cells up to 15 μ in diameter, the terminal of which have one small projecting papilla (or occasionally up to three or four); hairs agglutinated and forming a dense layer, not developing in tufts, decreasing downward and disappearing near base of perithecium, gradually changing upward into the small, slender, filiform, tapering, 1-3-celled hairs that form a dense layer over the short, broad, papilliform neck; wall thin, semitransparent, light-brown, composed of rather large swollen cells that reach a diameter of 20 μ. Asci eight-spored, cylindrical, 100-110 × 11-13 μ, broadly rounded above, indistinctly perforate at apex, tapering below into a short stipe, surrounded by a layer of hyaline ventricose cells but with no paraphyses intermixed. Ascospores irregularly uniseriate, strongly curved so that one side is concave, 11-13 × 6-8 μ (sometimes less curved and then reaching a length of 17 μ), becoming dark brownish-black and opaque, with a small circular apical germ pore; primary appendage basal, clavate, hyaline, small, measuring about 5 × 1 μ. No secondary appendages.

Type: Isolated in culture from seeds of *Daucus carota* L. var. *sativa* DC., Gilroy, Calif., U.S.A. Crypt. Herb. Dept. of Botany, Univ. of Toronto *22439*. Additional specimens from same isolation in Herb. Div. Botany and Plant Pathology, Dept. of Agr. Ottawa, DAOM *20504* and in Herb. RFC *12075*.

Sordaria curvispora has been isolated from seeds of *Apium graveolens* L. var. *dulce* DC. (celery) and *Daucus carota* L. var. *sativa* DC. (carrot). Both isolates were from seed from California.

This species, like the preceding, seems to be related to *S. fimbriata*, being distinguished by having the spores smaller and more distinctly curved with a slightly smaller primary appendage. The terminal cells of the agglutinated hairs are less fimbriate.

Occasionally the primary appendage is not separated from the spore by a septum and in that case becomes brown.

The perithecial initial develops from a coiled ascogonium (Fig. 16) that is surrounded by irregular hyphae that appear to grow out from cells at the base of the ascogonial cell.

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A DIAGRAMMATIC SCALE FOR ESTIMATING RUST INTENSITY ON LEAVES AND STEMS OF CEREALS[1]

By R. F. Peterson,[2] A. B. Campbell,[3] and A. E. Hannah[4]

Abstract

The Cobb scale and the modified Cobb (U.S.D.A.) scale were devised for estimating, by means of diagrams, the proportion of the area of a leaf or stem occupied by rust pustules. Existing diagrams illustrating these scales are inadequate in being arranged in intervals that are too large and irregular, and in not depicting a sufficient range of pustule sizes. New diagrams are presented using smaller regular intervals of rust intensity and a greater range of pustule sizes, thus providing a basis for more objective and accurate determinations.

Introduction

Diagrammatic scales are necessary for measuring rust intensity on leaves and stems of cereals. Such scales have been devised and published; and two of these, known respectively as the Cobb scale and the modified Cobb scale, have been widely used. Existing diagrams illustrating these scales, however, show certain inadequacies. The new diagrams here presented represent an attempt at improvement.

The first diagrammatic rust scale was published by Cobb (2) in 1892, and is reproduced in the present paper in Fig. 1. A full quotation of Cobb's description of this scale follows.

> The following figures illustrate the scale of rustiness devised and used for this report. The scale is in five parts, representing five degrees of rustiness. The gray spots are to represent the rust, and the oblongs on which they occur represent a portion of the wheat leaf, one and a half inches long. The lowest degree of rustiness is that where one per cent of the surface of the flag or sheaths is covered by rust. The next highest degree is where five per cent of the surface is covered with rust. The three other degrees are where ten, twenty, and fifty per cent of the surface is covered. I found this scale very easy of application. A dozen sample leaves were gathered at random; these were then passed one at a time over the above scale of drawings, when it was very easy to see which number of the scale was nearest to each leaf in turn, and the result was entered in the notes accordingly, the plus and minus signs being used to indicate a slightly greater or slightly less amount of rust than that indicated by the scale number. Thus, 20 + would mean an amount of rust which covered slightly more than twenty per cent, of the surface, and 20 − an amount which covered slightly less than twenty per cent. I have found this scale so convenient and so much more expressive and accurate than the indiscriminate use of terms like "very rusty", "quite rusty", "rotten with rust", &c., &c., that I have reproduced here my original figures, and hope to see them made use of by others. I believe that the numbers are more expressive than any words, but would call attention to the fact that the terms "very slightly", "slightly", "considerably", "very considerably", "exceedingly" have been used in this report to correspond with the above five degrees of rustiness.

It should be noted that in Cobb's diagrams the percentages given refer to the actual percentage of the leaf area occupied by rust pustules, and that although the highest reading shown in his diagrams is 50%, Cobb did not

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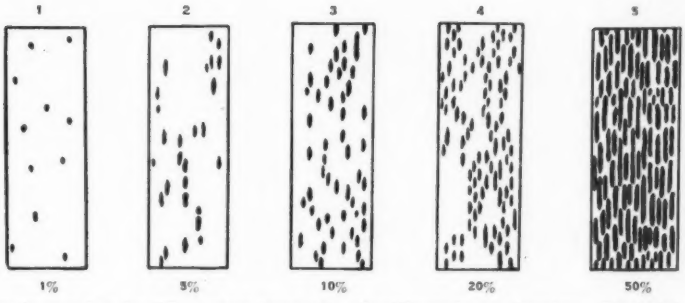
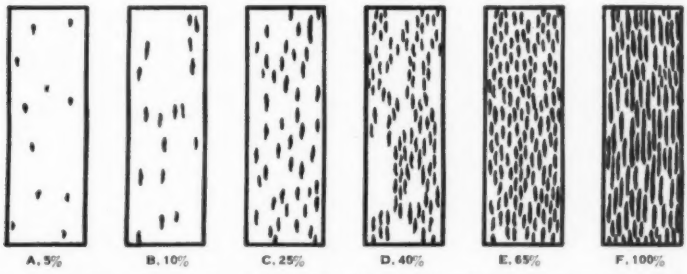
PLATE I



Five pieces cut from five different wheat leaves to show five different degrees of rustiness. The shaded spots represent rust.

FIG. 1. *Scale for estimating rust and caption as published by N. A. Cobb. (Reproduced from the Agricultural Gazette of New South Wales by permission of the Editor).*



Scale for estimating rust, illustrating six degrees of rustiness used in estimating the percentage of stem-rust infection. The shaded spots represent rust, and the figures represent approximately the rust percentages computed on the basis of the maximum of surfaces covered by rust as shown in the 100 per cent figure (F). Figure F in the diagram represents 37 per cent of actual rust-covered surface and is arbitrarily selected as 100 percent. The other percentages are in terms of Figure F.

FIG. 2. *Scale for estimating rust and caption as published by Leo. E. Melchers and John H. Parker. (Reproduced from U.S. Dept. Agr. Bulletin No. 1046 by permission of the Chief of Publications, U.S. Dept. Agr.).*

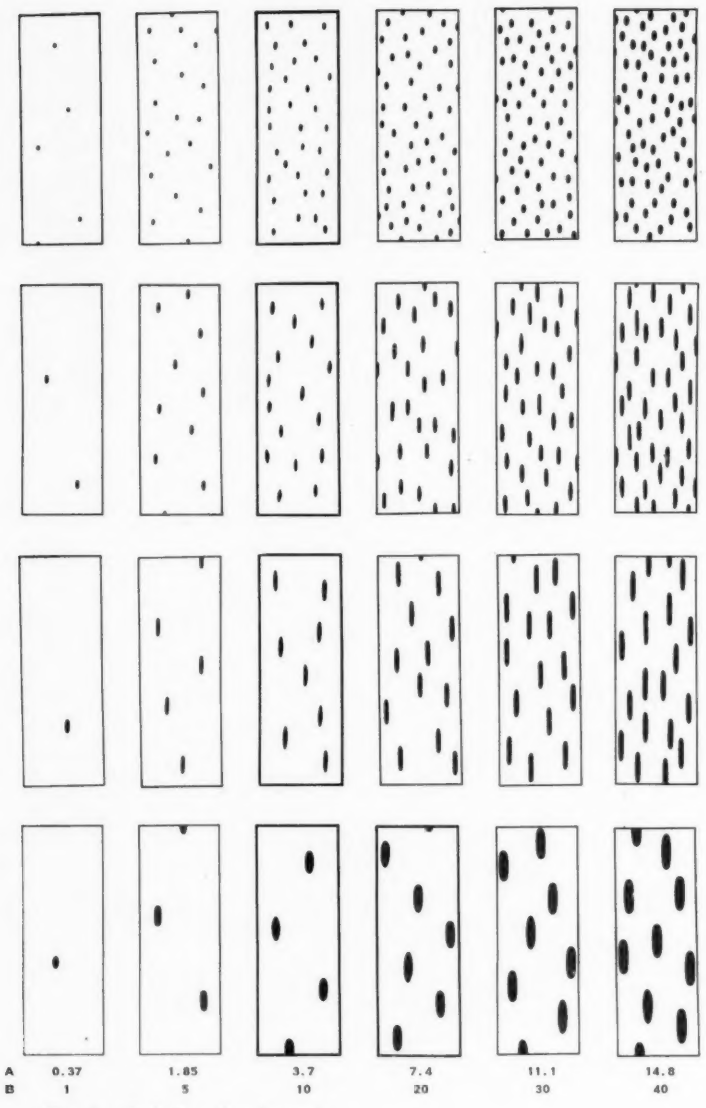


FIG. 3. *Scale for estimating rust.*
(*A*). *Actual percentage of area occupied by rust pustules.*
(*B*). *Standard rust readings according to the U.S.D.A. scale.* (*See Fig. 2*).

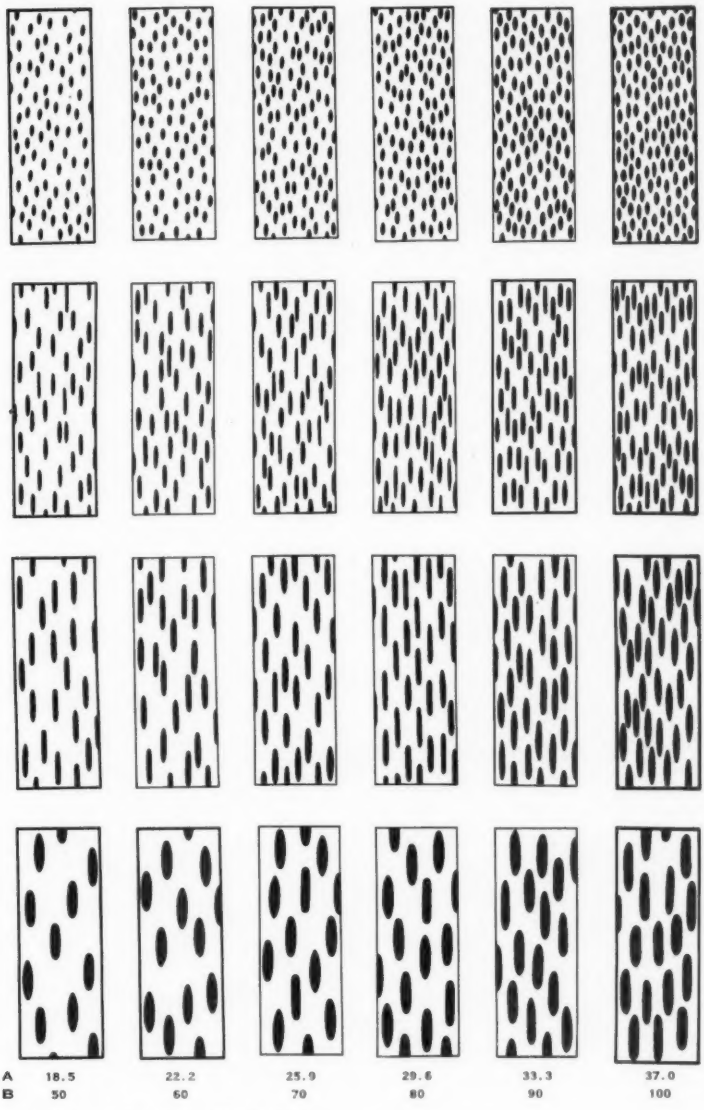


FIG. 4. *Scale for estimating rust.*
(A). Actual percentage of area occupied by rust pustules.
(B). Standard rust readings according to the U.S.D.A. scale. (See Fig. 2).

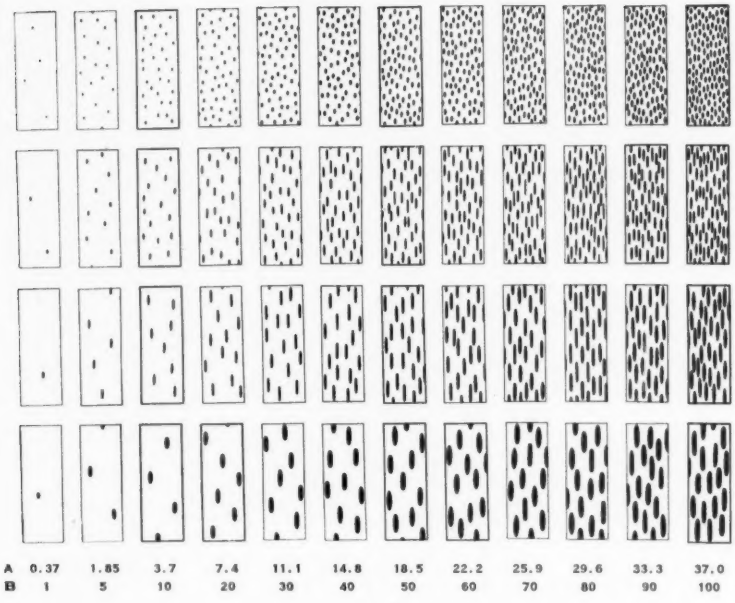

A 0.37 1.85 3.7 7.4 11.1 14.8 18.5 22.2 25.9 29.6 33.3 37.0
B 1 5 10 20 30 40 50 60 70 80 90 100

FIG. 5.

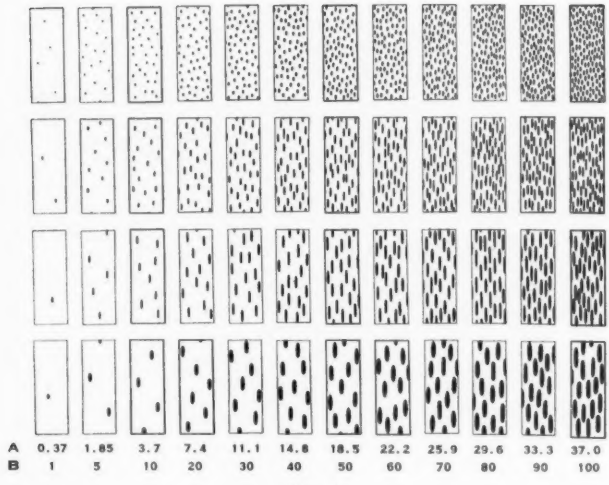

A 0.37 1.85 3.7 7.4 11.1 14.8 18.5 22.2 25.9 29.6 33.3 37.0
B 1 5 10 20 30 40 50 60 70 80 90 100

FIG. 6.

state or imply that this is a maximum reading. An investigator using Cobb's scale can, if he wishes, estimate rust percentages above 50%. The limit is obviously 100%, with the entire surface occupied by pustules. (This condition is sometimes attained on relatively small areas of leaves or stems of cereals where pustules have coalesced, but probably never on an entire leaf or stem). Cobb's scale then, if fully extended, provides for the estimation of the percentage of the area of a leaf or stem occupied by rust pustules through the full range from absence of rust to complete coverage of an area by rust pustules.

In 1917 the Office of Cereal Investigations of the Bureau of Plant Industry, United States Department of Agriculture, adopted a modification of the Cobb scale that was published by Melchers and Parker (4) in 1922. It is reproduced in the present paper in Fig. 2 and will be referred to here as the U.S.D.A. scale. In the development of this scale the diagram representing 37% of the total surface as occupied by rust pustules was arbitrarily selected as 100%, and the lower percentages were arranged in terms of this diagram. This procedure is based on the fact that mycelial development is more extensive than pustule development. The assumption is made that, in general, when pustules cover 37% of the surface, the development and destructiveness of the underlying mycelium are almost at a maximum. Supporting this assumption is the common experience that in the great majority of cases of cereal plants killed or badly damaged by rust, the total area of pustules on stems or leaves does not exceed 37% of the total area of those parts. The relatively few cases that exceed 37% are customarily classed as 100% when the U.S.D.A. scale is used.

The manner in which the originators of the U.S.D.A. scale derived their diagrams from those of the Cobb scale will be apparent from an examination of Figs. 1 and 2. Cobb's diagram having 50% of the total area shaded was apparently modified by removing enough of the shaded areas to leave 37% of the total area shaded. This was arbitrarily selected as 100%. The remaining diagrams were modified in a similar manner in order to have all percentages in terms of the arbitrary 100%. A new diagram (65%) was added.

Rust investigators have used the U.S.D.A. scale not only for classifying rusted plants in the six classes shown in Fig. 2, but also for estimating other percentages. Thus, in the bulletin by Melchers and Parker (4) the rust reactions of varieties are recorded by units from zero to five, and from there to 100 mainly in intervals of five, although smaller intervals are used in some cases. It appears that most other workers who have used the U.S.D.A.

EXPLANATION OF FIGURES

FIG. 5. *Scale for estimating rust. Reduced reproduction of Plates II and III.*
(A). Actual percentage of area occupied by rust pustules.
(B). Standard rust readings according to the U.S.D.A. scale. (See Fig. 2).

FIG. 6. *Scale for estimating rust. Reduced reproduction of Plates II and III.*
(A). Actual percentage of area occupied by rust pustules.
(B). Standard rust readings according to the U.S.D.A. scale. (See Fig. 2).

scale have recorded rust percentages in intervals of either 5 or 10, but sometimes in intervals of one, particularly in the low range from zero to five.

The U.S.D.A. scale has become the most commonly used basis for recording rust intensity on cereals in North America and is widely used in various countries throughout the world. The diagrams (Fig. 2) have been a great help to rust investigators but are inadequate for present-day use (as are also the original diagrams of Cobb) in the following respects:

1. Too few diagrams are shown, making a great deal of interpolation necessary when recording rust readings in intervals of 5 or 10%.
2. The irregular intervals used make classification difficult.
3. The depicted pustules do not adequately represent the great range in size of uredia and telia of rusts occurring on cereals.

The new diagrams here presented (Plates II, III, and IV) have been developed in an attempt to overcome, to some extent, the inadequacies of the older diagrams. They are intended for use in connection with uredia and telia of the following rust species:

Puccinia graminis Pers.
P. rubigo-verà (DC.) Wint.
P. hordei Otth.
P. coronata Cda.

The writers do not consider these diagrams suitable for stripe rust caused by *P. glumarum* (Schm.) Eriks. and Henn.

The New Diagrams

Large scale diagrams (19.3 × 7 in.) representing portions of leaves or stems of cereal plants were drawn on white showcard; and areas representing rust pustules were outlined in pencil and filled in with India ink. A planimeter was used to measure the areas of the shaded portions of the diagrams. To represent 100% of rust on the basis of the U.S.D.A. scale the total area of pustules was arranged to be 37% of the area of the diagram, and lower percentages in the remaining diagrams were arranged in proper proportion. With the size of diagram used, 1% on the basis of the U.S.D.A. scale (0.37% of the total area) is 0.5 sq. in., a convenient unit for planimeter readings.

Instead of a single series of five or six diagrams as shown in Figs. 1 and 2, four series of diagrams with different pustule sizes were prepared, each series having 12 diagrams. After all pustule areas had been checked with the planimeter, individual photographs were made of the 48 diagrams and these were mounted in proper order in four series to be photographed again and reproduced as a single figure. The resulting scale is shown in Plates II and III. In order to provide a still greater range of pustule sizes this scale is reproduced in two different reductions in Figs. 5 and 6. Other reductions or magnifications of this scale can readily be made if this should be found desirable.

Both the actual percentages corresponding to the Cobb scale and the readings according to the U.S.D.A. scale are shown, so that the present scale is a dual purpose one. It was not possible to make this scale equally convenient (reading in multiples of 5 or 10) for both types of readings. It has been arranged to be most convenient for the readings corresponding to the U.S.D.A. scale, because this is at present the more commonly used of the two scales. The four horizontal series of diagrams are arranged so that the four diagrams making up any column have equal total areas of pustules.

The method of using the new diagrams in Plates II, III, and IV is to select a diagram in which the pustules most closely resemble, both in size and in density of spacing, the pustules on the leaf or stem under study. The reading given below the selected diagram will be the estimate of the proportion of the area of the leaf or stem that is occupied by pustules. A more objective and accurate reading is obtained in this way than by using only diagrams depicting pustules of average size. This point will be readily appreciated if the reader will examine the four diagrams in a given column (say the last column in Plate II) and the corresponding eight diagrams in Plate IV. Although each of these 12 diagrams has 14.8% of its area shaded, the visual impression of the proportion of area shaded varies from one diagram to another owing to changes in pustule size.

One of the difficulties encountered in using the older scales has been the classifying of leaves carrying extremely small pustules of *P. rubigo-vera*, *P. hordei*, or *P. coronata*. The writers have found the upper row of diagrams of Figs. 5 and 6 useful for cases of this kind.

Pustules differing markedly in size are sometimes found on the same leaf or stem. It has not been attempted here to represent pustules of varying size within individual diagrams because of the large number of possible combinations of pustule sizes. In actual practice it is often found that pustules of a certain size predominate in a given area of leaf or stem, and this facilitates the use of diagrams of the type provided.

Discussion

It is not proposed to describe here the various published diagrammatic rust scales, nor to discuss the factors other than rust intensity (e.g., uredial infection type and stage of crop development) that must be considered in estimating crop loss due to rust. Chester (1) has recently published a discussion and bibliography dealing with these points, and Dickson (3) has illustrated the uredial infection types. The scope of the present paper is limited to the estimation of the proportion of the area of a given section of leaf or stem occupied by rust pustules.

A given reading in terms of the U.S.D.A. scale may be converted into an actual percentage reading by multiplying by the factor 0.37. Similarly, a given reading in actual percentage may be converted into a reading in terms of the U.S.D.A. scale by multiplying by the factor 2.7027. These relationships are true only in those cases where the area occupied by pustules does not

exceed 37% of the total area under consideration. Higher rust intensities than this are not distinguished by the U.S.D.A. scale, since it is customary to class them all as 100% rather than use numbers over 100.

Even if increases in rust intensity beyond the 37% point may have little significance in resultant destructiveness, most investigators would wish to be in a position to take such increases into consideration in critical studies. Thus the Cobb scale has an undoubted advantage over the U.S.D.A. scale in being more widely applicable in scientific work.

In most papers recording rust readings based on the U.S.D.A. scale the readings are below 100 so that the complication described above does not arise. In some papers, however, readings of 100 appear, and one is not certain whether the actual percentage is 37 or more. If investigators using the U.S.D.A. or similar scales would record estimates of the actual percentages represented by their relatively few recorded scores of 100, this complication could be avoided in all cases. The last diagram in Cobb's scale (Fig. 1) will aid in making such estimates.

Acknowledgment

The writers are indebted to Mr. W. E. Clark, Technician, Dominion Laboratory of Plant Pathology, Winnipeg, for photographing all diagrams shown in Plates I to IV inclusive.

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